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Salt and the Rough-skinned Newt (Taricha granulosa): Evolutionary Investigations of Local Adaptation to an Anthropogenic and Natural Stressor

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SALT AND THE ROUGH-SKINNED NEWT (*TARICHA GRANULOSA*): EVOLUTIONARY INVESTIGATIONS OF LOCAL ADAPTATION TO AN ANTHROPOGENIC AND NATURAL STRESSOR

by

Gareth Rowland Hopkins

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ABSTRACT

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Gareth R. Hopkins, Doctor of Philosophy

Utah State University, 2015

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Department: Biology

Amphibians are osmotically-challenged due to their highly permeable skin and egg membranes. As such, biologists have mostly ignored their occurrence in saline habitats. The goal of this dissertation was to investigate the ability of amphibians to adapt to inhabit these osmotically-stressful environments. As freshwater resources are increasingly salinized due to a combination of anthropogenic and natural stressors, including road-deicing salt application, coastal storm events, and sea-level rise, it is important to understand the abilities and constraints of sensitive organisms, like amphibians, to persist in these environments. I began by exploring what is known about amphibians inhabiting saline habitats around the world, and reviewed information dating from the 1800s to the present, documenting over 100 species inhabiting inland and coastal environments. I then proceeded to investigate the evolutionary potential for one

species in particular, the rough-skinned newt (*Taricha granulosa*) to adapt to increased salinity in its habitat. I documented the negative effects of salinity (both NaCl and $MgCl_2$ – the two most commonly-used road deicers) on embryonic and larval survival and development through a series of experiments, and explored the importance of both developmental and evolutionary history with a stressor on survival. I investigated the evolutionary potential of salt-naïve populations through examining intrapopulation and interfamily variation in critical early life-history traits, and determined that there is high interfamily variation in the salt tolerances of offspring of individual females within a population, providing the raw material for natural selection and local adaptation. Finally, I discovered a population of newts living in the tidal area of a coastal stream, with a natural source of salinity. Through conducting a series of laboratory salt challenges and assays on osmotic, stress, and immune physiology, I was able to determine that newts in the tidal area appear to be physiologically adapted to increased salinity compared to newts in a freshwater area upstream. All this information suggests that amphibians, while still osmotically-challenged, may also not be helpless in the face of salinization, and populations may be able to locally adapt to habitats impacted by natural and anthropogenic sources of salinity.

(362 pages)

PUBLIC ABSTRACT

Salt and the Rough-skinned Newt (*Taricha granulosa*): Evolutionary Investigations of Local Adaptation to an Anthropogenic and Natural Stressor

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Amphibians are generally considered intolerant of salt water, due to their permeable skin, and jelly-cased eggs. As such, biologists have mostly assumed they could not live in saline habitats. As freshwater habitats are increasingly salinized due to road-deicing salt application, coastal storm events, and sea-level rise, it is important to understand the abilities and constraints of animals such as amphibians living in these habitats to adapt and persist. The goal of this dissertation was to investigate the evolutionary ability of amphibians to adapt to inhabit saline environments. I began by exploring what is known about amphibians inhabiting saline habitats around the world, and reviewed information dating from the 1800s to the present, documenting over 100 species inhabiting inland and coastal environments. I then proceeded to investigate the potential for one species in particular, the rough-skinned newt (*Taricha granulosa*) to adapt to increased salinity in its habitat. I discovered through a series of experiments that salt can have serious negative effects on newt egg and larval survival and development,

and that the type of salt used (NaCl versus $MgCl_2$ – the two most commonly used road deicers) can be important in predicting survival. However, I also discovered that there was a lot of variation in salt tolerance of eggs among females from the same population. This variation is what natural selection can act upon, and therefore it appears that newt populations may be able to locally adapt to increased salinity in their habitats from road deicing salts. Finally, I discovered a population of newts living in the tidal area of a coastal stream with a natural source of salinity. By comparing the physiological responses to salt of animals from the tidal area versus those found in an upstream freshwater area, I was able to determine that newts in the tidal area appear to be physiologically adapted to increased salinity. All this information suggests that amphibians, while still sensitive to salt water, may also not be helpless in the face of salinization, and populations may be able to locally adapt to habitats impacted by natural and human sources of salt.

to

The Newts

and to

Doc

who loves them most of all

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Gareth R. Hopkins

CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

"Common salt is poison to the Amphibia."

- Gadow (1901). *Amphibia and Reptiles*

"Nature provides exceptions to every rule."

- M. Fuller (1810-1850)

CHAPTER 1

INTRODUCTION

The world is a stressful place. Organisms face countless natural stressors in their habitats, from temperature fluctuations to aridity, food scarcity, the need for oxygen and water, and threats from competitors and predators. Increasingly, however, organisms also have to cope with anthropogenic stressors, from a local scale, such as pollution (in its myriad forms) and habitat destruction/degradation, to a global scale, such as increased UV radiation due to planetary ozone layer degradation, and unpredictable weather and temperature patterns due to climate change.

My research interests lie in the ability of organisms to evolutionarily respond and adapt to these stressors, whether they be natural or anthropogenic. Understanding the ability, and constraints, of organisms to adapt to stressful environments allows us to investigate a critical question: how can organisms adapt to the anthropogenic change that has, does, and will continue to, dominate the ecosystems of this planet? While much research in environmental science, ecotoxicology, and conservation biology has focused on the important negative effects of stressors (as will an aspect of this dissertation), the ability of organisms to evolve in response to these intense selective pressures has been less studied.

This dissertation explores this broad topic using a particular stressor and organism as a case study of understanding evolution in stressful environments. I chose an abiotic stressor that is both natural and anthropogenic in nature, salinity, and an osmotically-

sensitive organism, an amphibian, the rough-skinned newt (Amphibia: Caudata: Salamandridae, *Taricha granulosa* Skilton) as the biological subject.

Rough-skinned newts (*Taricha granulosa*) (Fig. 1.1) are western North American caudate amphibians with a wide distribution, inhabiting ponds, streams and lakes from California to Alaska (Nussbaum, Brodie & Storm 1983). They are found in mountain ponds, 2800m in elevation, where road deicing salts are applied in the winter, down to sea-level (Nussbaum et al. 1983), where a population was anecdotally spotted in a tidal coastal stream in Oregon in the 1950s (Ferguson 1956). This brief anecdote is our only hint of possible salt tolerance in this species, and no formal work has been completed on the effects of salinity on *Taricha*.

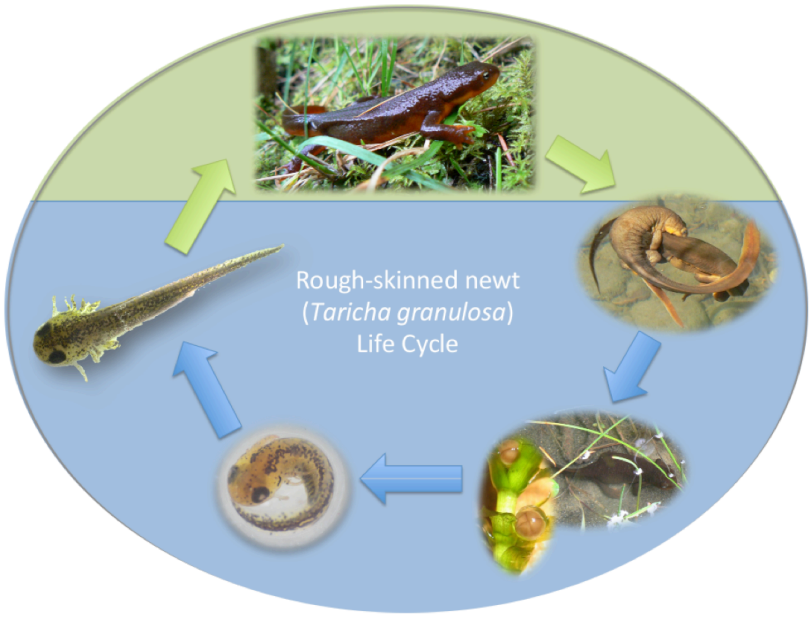

Fig. 1.1. Life cycle of the rough-skinned newt (*Taricha granulosa*). Adult newts spend the majority of their life terrestrially, returning to aquatic environments to breed in the spring. Mating occurs in the water and fertilized eggs are typically deposited on aquatic vegetation, where they develop over the next month. Aquatic larvae hatch and develop for several months in the water before metamorphosing and leaving the water.

In this Introduction I will first review the stressful nature of salts, both anthropogenic and naturally-sourced, on amphibians, and why salinity is such an important and timely stressor to examine. I will then give a primer on the basics of local adaptation and the evolutionary concepts we must understand and examine to explore the potential for adaptation in stressful environments.

Amphibians: An Osmotically Challenged Group of Animals

Amphibians, having highly permeable skin and egg membranes, are an osmotically-challenged group of animals (Shoemaker & Nagy 1977). Adult tree frogs, for instance, have been recorded to lose, through evaporative passive water flux, up to 250 ml body water/kg/day, far exceeding the less than 3 ml/kg/day loss of most reptiles (Shoemaker & Nagy 1977). At the same time, amphibian skin can be very efficient at transporting Na^+ and Cl^- ions into the body (Duellman & Trueb 1994). Having this sort of osmotic physiology, it is not surprising that amphibians have long been viewed as extremely intolerant of salt (Darwin 1872; Gadow 1901). Experimentally, salt solutions have been found to retard growth and development, lead to increased developmental deformities and physiological stress, and even kill all life stages of amphibians (Ely 1944; Ruibal 1959; Beebee 1985; Padhye & Ghate 1992; Viertel 1999; Turtle 2000; Chinathamby et al. 2006; Dougherty & Smith 2006; Karraker & Ruthig 2009; Langhans 2009; Chambers 2011; Duff 2011; Harless et al. 2011). In the field, the presence of salt in habitats can influence amphibian demography and community structure (Karraker, Gibbs & Vonesh 2008; Collins & Russell 2009) and many species show marked avoidance of saltwater habitats (Davenport & Huat 1997; Viertel 1999; Haramura 2008;

Haramura 2011). Despite this sensitivity, high concentrations of salt, from either anthropogenic or natural sources, are often found in amphibian breeding habitats, and cannot be avoided (e.g., Beebee 1985; Environment Canada 2001).

Anthropogenic Sources of Salt

Humans are salinizing the world's freshwater resources in both the southern and northern hemispheres (Cañedo-Argülles et al. 2013). In the southern hemisphere, this is epitomized in much of Australia, where secondary salinization of inland freshwater system due to agricultural waste, increased aridity as a result of climate change, and landscape modification, is becoming a significant problem (Williams 2001), especially for the continent's amphibians (Christy & Dickman 2002; Chinathamby et al. 2006; Kearney, Byrne & Reina 2012). In the northern hemisphere, in both North America and Europe, anthropogenic salinization is mostly due to the application of road deicing salts (Environment Canada 2001; Thunqvist 2004; Kaushal, Groffman, Likens et al. 2005; National Transportation Research Board 2007), and this source of anthropogenic salt will be the focus of much of this dissertation.

A dramatic salinization of North America's fresh water has been occurring over the last three decades, principally as a result of road deicing salt application (Kaushal et al. 2005). Approximately 14 million tons of salts are applied to roads across North America every winter, and the run-off from roads has led to the salinization of road side ponds of up to 4000-5000 mg/L (4-5 ppt) Cl^-, a limit far exceeding the 220 mg/L (0.22 ppt) deemed harmful to at least 10% of aquatic life (Environment Canada 2001). Numerous studies have found that Cl^- concentrations in roadside ponds greatly exceed

those in ponds located away from roads (Turtle 2000; Karraker et al. 2008; Collins & Russell 2009; Brady 2012). Roads cover approximately 1% of the land area in the contiguous United States (Forman 2000), and it has been estimated that 20% of land area is located within 127 m of a road (with only 3% located over 5 km away) (Riitters & Wickham 2003). This sort of prevalence and proximity has direct ecological consequences (Forman 2000; Trombulak & Frissell 2000), and amphibian species richness has, not surprisingly, been negatively correlated with road density on the landscape (Houlahan & Findlay 2003; Collins & Russell 2009).

Amphibians have been shown to be severely affected by road deicing salts, with increased concentrations of NaCl causing egg, larval and adult mortality, impaired growth and development, and deformities (Viertel 1999; Turtle 2000; Dougherty & Smith 2006; Karraker 2007; Collins & Russell 2009; Karraker & Ruthig 2009; Langhans 2009; Duff 2011; Harless et al. 2011). Recently, $MgCl_2$ has started to be used by government agencies alongside or in place of NaCl, and is now the 2nd most commonly used road salt in North America, after NaCl (National Transportation Research Board 2007). Although NaCl may still be the most abundantly applied salt, a study examining the leaching of road deicing salts in soils in New York State found that Mg^{2+} from $MgCl_2$ was the most abundant reactive salt cation in roadside soils (Cunnigham 2008). In addition, $MgCl_2$ contains 74.7% Cl^- compared to the 60.66% of NaCl (National Transportation Research Board 2007), indicating that its environmental effects may be proportionally larger than its use. The only two published studies on the effects of $MgCl_2$ deicing salts on amphibians have both indicated that $MgCl_2$ may actually be more toxic than NaCl to anuran tadpoles (Dougherty & Smith 2006; Harless et al. 2011), and even some species

that are relatively tolerant of NaCl are intolerant of $MgCl_2$ (Dougherty & Smith 2006). However, the effects on amphibian eggs, or any life stage of caudates (salamanders and newts) are unknown.

Globally, climate change is resulting in a dramatic increase in the frequency of storm events (Trenberth 2011), which could directly affect the application of deicing salts in the northern hemisphere. Mean increases in temperature could also lead to drought and the desertification of many areas in the world (Trenberth 2011), increasingly the number of saline temporary ponds and streams in arid environments, adding more of these "naturally" saline habitats for amphibians to deal with (see below). In addition, rising sea levels and increased storm surges could lead to the increasing salinization of coastal freshwater ecosystems (Gornitz 1995; Nicholls, Hoozemans & Marchand 1999; Lowe & Gregory 2005; Purcell et al. 2008; Rios-López 2008), which could directly threaten amphibians living in coastal areas with a "natural" source of salinity (Rios-López 2008; Alexander et al. 2012).

Natural Sources of Salt

The oceans of the world are arguably the most common source of natural salt, and thus a variety of coastal ecosystems where amphibians exist have the potential to be impacted by natural salinity. These range from mangrove swamps in the tropics, impacted by daily predictable tidal salt inundations (e.g., Jena, Palita & Mahapatra 2013), to salt marshes with constantly fluctuating salinity (e.g., Christman 1974) to tidal streams, ponds, and pools impacted by unpredictable storm surges and sea-spray along the rocky coasts of much of northern North America and Europe (e.g., Pyefinch 1937; Spurway

1943; Roberts 1970), and beaches throughout the world (Storer 1925; Bellairs & Shute 1954). Vernberg and Vernberg (2001) provide a good background on the geography and biogeochemistry of these "coastal zone" habitats, and Chapter 2 of this dissertation is focused on reviewing the known literature of amphibians inhabiting these, and other saline habitats around the world. Suffice to say, at the present moment, although osmotically challenging environments, certain amphibians around the world have found ways to colonize many of these coastal habitats (see Chapter 2). This is in direct contrast to the prevailing view of most biologists, who have long viewed these habitats as unsuitable for amphibians due to their high oceanic salt concentrations, and the well-known osmotic sensitivities of amphibians. Darwin (1859, p. 393) himself explained the apparent absence of amphibians on many of the world's islands in *The Origin of Species* (1st ed.) by the statement: "these animals and their spawn are known to be immediately killed by sea-water."

Inland from the coastal zone, naturally saline habitats where amphibians might be found include salt lakes (e.g., Brandon, Maruska & Rumph 1981), arid environments with salt flats (e.g., Cei 1955; Ruibal 1962) and temporary streams and ponds (e.g., Gomez-Mestre & Tejedo 2003), and saline hotsprings (e.g., Brues 1932). These all represent challenging, osmotically stressful environments for amphibians.

Local Adaptation and the Nature of Evolution by Natural Selection

Whether due to anthropogenic (Chapters 4-6) or natural (Chapter 7) sources of salt, amphibian populations that manage to survive and breed in saline waters may do so because they have evolved to be locally adapted to that harsh environment (Gomez-

Mestre & Tejedo 2003; Brady 2012). Local adaptation is simply the set of evolved traits in a local population that provide an advantage for that population under those specific environmental conditions, regardless of the fitness consequences of those traits in other habitats (Kawecki & Ebert 2004). Regarding evolution, Darwin (1859) wrote in *The Origin of Species* that "we see nothing of these slow changes in progress, until the hand of time has marked the long lapse of ages." (p. 84). This perspective that evolution acts at a very slow, almost unobservable pace has traditionally dominated the thinking of most ecologists and conservation biologists, and as such, the ability of organisms to evolutionarily respond and adapt to stressors has been mostly ignored. Recently, however, we have begun to realize that evolution can occur quickly, on ecological time scales (often in less than 10 generations), in response to environmental stressors (Kawecki & Ebert 2004; Carroll et al. 2007), and that organisms are not nearly as helpless in the face of stressors as has been assumed.

Regardless of the pace of evolution, if it acts in response to anthropogenic or natural stressors, the basis for local adaptation is natural selection (Darwin 1859; Kawecki & Ebert 2004). If we are to understand the ability of organisms like amphibians to adapt to stressful environments, we must first therefore understand the fundamental requirements of natural selection. First, individual variation in traits must exist in a population of individuals (explored in Chapter 3 of this dissertation). Second, that variation must be heritable. Finally, variation in traits must confer fitness advantages in certain environments to individuals with certain variants of the trait over other individuals with other, less desirable variants. In other words, alleles must exist that improve fitness in a habitat in which most individuals perform poorly (Kawecki & Ebert 2004). While it

has been established that saline environments are indeed such habitats for most amphibians, this critical question of the amount, and nature of variation in how poorly, or well, individuals within a population do in these habitats is virtually unknown (explored in Chapter 4 of this dissertation). If a trait is to evolve, the selective pressure on that trait must be great (explored in-depth in Chapters 4-6), and one would expect that individuals surviving selection would be more adapted to their local environment (explored in Chapter 7).

It is this dissertation's goal to bring an evolutionary perspective to the topic of amphibian salt tolerance, through grounding itself in these principles of natural selection. It explores both the intense nature of the selective pressure of salt by investigating the effects of this stressor on newt early life-history traits and survival, as well as the ability (and constraints) of populations to adapt to this natural and anthropogenic stressor. Such an understanding of the evolutionary potential for salt tolerance in amphibians is both fundamental to evolution and biogeography, as it has been since Darwin's (1859) first musings on the subject, and critically timely for conservation, as the world's freshwater resources become increasingly saline, and amphibian populations continue to decline worldwide (Stuart et al. 2004; Mendelson et al. 2006).

Overview of Study Sites

Newts examined in the following chapters were collected from two main areas of Oregon (Fig. 1.2): a salt-naïve freshwater pond population at the Soap Creek Ponds near the town of Corvallis (Benton County) (Chapters 3–6), and newts found in both the tidal and upstream freshwater areas of a coastal, estuarine stream, Hunter Creek, near the town

of Gold Beach (Curry County) (Chapter 7). Details of study sites are presented in each chapter.

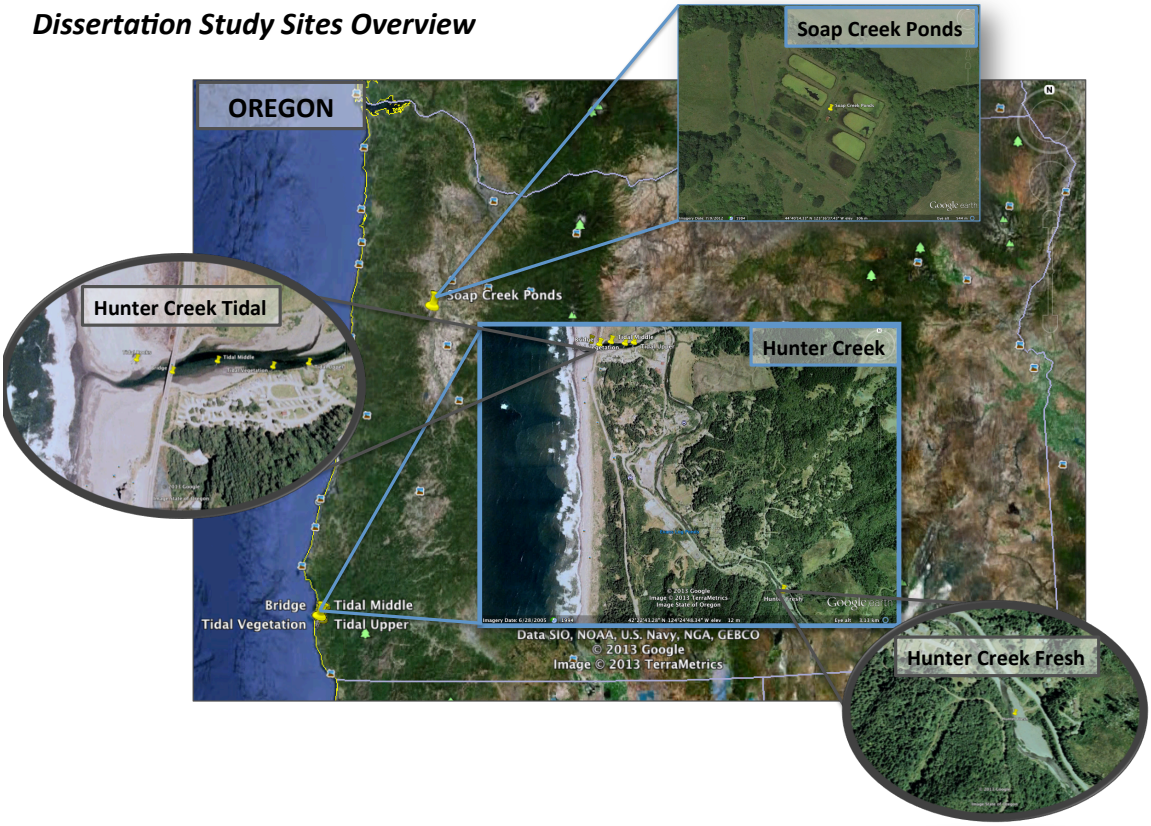

Fig. 1.2. Overview of study sites and newt populations throughout the State of Oregon used in this dissertation research.

Overview of Chapters

Chapter 2. This chapter provides an historical background on the subject of amphibian salt tolerance, and reviews the literature of amphibians inhabiting saline

environments. It makes the case that amphibian salt tolerance has evolved multiple times in many different families around the world, and is perhaps not as rare a phenomenon as previously thought. However, we still know very little about how this fascinating trait actually evolves in populations.
[partly in Hopkins, G.R., and E.D. Brodie, Jr. 2015. Occurrence of amphibians in saline habitats: a review and evolutionary perspective. *Herpetological Monographs* 29: 1-27.]

Chapter 3. This chapter examines the degree and nature of natural interfamily variation that exists in early life-history traits (time to hatching, size, and developmental stage at hatching) among newt families from the same population. Variation was assessed over two years and two developmental temperatures. There was significant variation among the offspring of different females regardless of these factors, and we could also not explain the variation by any maternal effects. This leads us to conclude that there is sufficient genetic variation in early life-history traits, important for fitness, within a population of newts for natural selection to act upon.
[Hopkins, G.R., Gall, B.G., French, S.S. and E.D. Brodie, Jr. 2012. Interfamily variation in amphibian early life-history traits: raw material for natural selection? *Ecology and Evolution* 2: 1637-1643.]

Chapter 4. This chapter explores salt as an agent of natural selection to act upon the variation in early life-history traits elucidated in Chapter 3. Specifically, we examined the effect of the two most commonly used road deicing salts, NaCl and $MgCl_2$, on survival and embryonic development of newt eggs from an inland, salt-naïve

population. We document that both salts increase mortality of eggs in a dose-dependent fashion, and for those eggs that it did not kill, caused to hatch early, and at a reduced size and developmental stage. Thus, salt has the potential to act as a powerful agent of selection. Importantly, however, we also found significant variation in salt tolerance among eggs from different females, the raw material for natural selection, indicating that there is the evolutionary potential for local adaptation to increased salinity in a salt-naïve population of newts.

[Hopkins, G.R., French, S.S. and E.D. Brodie, Jr. 2013. Potential for local adaptation in response to an anthropogenic agent of selection: effects of road deicing salts on amphibian embryonic survival and development. *Evolutionary Applications* 6: 384-392.]

Chapter 5. This chapter serves as a sister chapter of Chapter 4 and further documents the destructive nature of salts (both NaCl and $MgCl_2$) on newt early life-history traits, specifically by examining the developmental deformities that occur as a result of salt exposure. We develop a novel scoring system for the severity of deformity, to clarify the actual negative power of salt as a selective agent, as not all deformities are equal in their severity.

[Hopkins, G.R., French, S.S., and E.D. Brodie, Jr. 2013. Increased frequency and severity of developmental deformities in rough-skinned newt (*Taricha granulosa*) embryos exposed to road deicing salts (NaCl & $MgCl_2$). *Environmental Pollution* 173: 264-269.]

Chapter 6. This chapter elucidates the significant carry-over effects of embryonic exposure to salt, documented in Chapters 4-5, on larval newt survival, post-hatching in

salt water. In doing so, we discuss the importance of documenting the effects of a stressor over life-history transitions, as its effects can be long-term in nature. We also discover a possible constraint on amphibian evolution in particular types of salt water in this chapter; whereas the effects of $MgCl_2$ on newt eggs were similar to that of NaCl, newt larvae have the ability to osmoregulate in NaCl, due to an evolutionary history with this stressor, but not large quantities of anthropogenically-derived $MgCl_2$, with which they lack this evolutionary history. It may be that amphibian populations have a better chance of adapting to NaCl as a road deicing salt than $MgCl_2$.

[Hopkins, G.R., Brodie, Jr., E.D., and S.S. French. 2014. Developmental and evolutionary history affect survival in stressful environments *PLoS ONE* 9: e95174.]

Chapter 7. This chapter switches gears from an anthropogenic to a natural source of salt: the ocean. Here we follow up on Ferguson's (1956) observation and document a population of newts living and breeding in the tidal area of a stream on the Oregon coast (as class as 100 m from the ocean). We compare the osmotic (body weight change, plasma osmolality, Na^+/K^+-ATPase), stress (the hormone corticosterone), and immune (bactericidal ability of plasma immune components) physiological response to laboratory salt stress of newts from the tidal area of the stream with those found in an osmotically stable freshwater area, upstream. We find that while increased salinity has effects on adult newt physiology in general, tidal newts generally show fewer disruptions from homeostasis compared to freshwater newts. Thus, there is some evidence that tidal newts may have physiologically adapted to living in this osmotically stressful environment.

[Hopkins, G.R., Brodie, Jr., E.D., Mohammadi, S., Brusch IV, G.A., Neuman-Lee, L.A., Hopkins, Z.M., and S.S. French. Tidal Newts: Physiological responses to an osmotically stressful environment [*submitted to Functional Ecology*]]

Chapter 8. This chapter summarizes the information gained about salt and the rough-skinned newt in this dissertation to help elucidate an evolutionary model of salt tolerance in amphibians. Avenues for future research on amphibian salt tolerance, and broad lessons learned for understanding evolution in stressful environments are discussed.

[partly in Hopkins, G.R., and E.D. Brodie, Jr. 2015. Occurrence of amphibians in saline habitats: a review and evolutionary perspective. *Herpetological Monographs* 29: 1-27.]

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CHAPTER 2

OCCURRENCE OF AMPHIBIANS IN SALINE HABITATS: A REVIEW[1]

Amphibians are well known as osmotically sensitive organisms due to their highly permeable skin and eggs, and, as such, biologists have mostly discounted their presence in saline environments. Yet, from the 1800s to the present day, scientists have repeatedly found amphibians living and breeding in a variety of saline coastal and inland habitats. Despite this plethora of observations, their presence in these habitats is still mostly ignored, and the last (and only) complete literature review documenting amphibians in brackish and saline habitats was completed over 50 years ago. Here we provide a review of the literature of amphibians in saline waters and present data on 144 species, in 28 families, on every continent except Antarctica. In doing so, we make the case that salt tolerance in amphibians may not be as rare as generally assumed. Through classifying habitats and studies, we conclude that the abilities of dozens of species to locally adapt to coastal and inland saline habitats have been extensively studied, although more work on most observed species is still needed.

[1] Coauthored by Gareth R. Hopkins and Edmund D. Brodie, Jr. Reprinted (in part) with permission of Allen Press from Herpetological Monographs Vol. 29, pages 1-27, 2015.

"These animals and their spawn are immediately killed (with the exception as far as known, of one Indian species) by sea-water."

— Charles Darwin (1872)

FOR NEARLY as long as biologists have been aware of amphibian intolerance of salt water, they have been fascinated by exceptions to this rule. Thus, in discussing the general lack of amphibians on islands, Darwin (1872) amended his statement on amphibian intolerance of salt water in the 6th edition of *Origin of the Species* to include the parenthetical exception "of one Indian species." "I may add," he wrote to Alfred Russel Wallace a few years later (probably referring to *Fejervarya cancrivora*), "that there is an Indian toad which can resist salt water and haunts the seaside." (Darwin 1876). While on the voyage of the Beagle in Port Desire, Patagonia, Argentina, in January 1834, Darwin noted that a "*Rana* …is bred in and inhabits water far too salt to drink" (Darwin 1834), a habitat Bell (1843) agreed was "remarkable" when identifying the frog as *Leiuperus salaries* (= *Pleurodema bufoninum*).

Since Darwin, countless other biologists and naturalists have found other frogs, toads, salamanders and newts "haunting the seaside," and remarked on these fascinating exceptions to the 'rule' of amphibian intolerance of salt water (Table 1). Neill (1958) compiled these anecdotes into his opus, "The occurrence of amphibians and reptiles in saltwater areas, and a bibliography." This paper is the only complete review of amphibians in saline habitats to date, and it includes mostly anecdotal notes of occurrence of amphibians in habitats impacted by salt water. At the time of its publication, very little

work had been completed on amphibian osmoregulatory physiology, including the now classic work of Malcolm S. Gordon and colleagues (e.g., Gordon et al. 1961); very few of the species mentioned in Neill's publication had been tested for salt tolerance, nor had the salinity of their habitats been measured. This pattern of what we would today call "natural history notes" makes up the bulk of the literature on amphibians and salinity, and it persisted as the norm from the 1800s to the early 1950s. Nevertheless, Neill's (1958) compilation of over 40 species of amphibians showing some evidence of salt tolerance provided the first glimpse that such tolerance may be more widespread than originally assumed.

In the 1960s and '70s, Malcolm S. Gordon (e.g., Gordon et al. 1961), Uri Katz (e.g., Katz 1973), Ronald H. Alvarado (e.g., Alvarado and Moody 1970) and others completed seminal osmoregulatory physiology studies on amphibians and their ability to regulate salts. Much of this work focused on the physiological ability of the Asian Crab-eating Frog, *Fejevarya* (= *Rana*) *cancrivora*, and the European Green Toad, *Bufotes* (= *Bufo*) *viridis* (= *balearicus*) to inhabit coastal habitats with salinities approaching that of full-strength seawater. Although many observers, including Darwin (1872), had long commented on the presence of these species in tidal mangroves, beaches, and in some cases, actually in the sea, Gordon, Katz, and their colleagues demonstrated experimentally how these animals achieved this remarkable tolerance. Their elucidations of the mechanisms of urea hypersynthesis and retention and Na^+ and Cl^- uptake to increase the osmolarity of the body fluids and plasma to be isotonic with the surrounding seawater (e.g., Gordon et al. 1961; Gordon 1962; Gordon and Tucker 1965,1968; Katz 1973,1975) are now considered classic works in amphibian physiology (reviewed by

Balinsky 1981; Katz 1989; Shoemaker et al. 1992). This mechanism has since been discovered in other salt-tolerant species, e.g., *Ambystoma tigrinum* (Kirschner et al. 1971; Romspert and McClanahan 1981; Gasser and Miller 1986), *Batrachoseps* spp. (Jones and Hillman 1978), *Rhinella marinus* (Liggins and Grigg 1985), *Epidalea calamita* (Gomez-Mestre et al. 2004), *Pseudacris regilla* (Weick 1980).

The remarkable finding that physiological adaptations allowed *Fejevarya cancrivora* in particular to survive in practically marine habitats with daily predictable sources of tidal salinity captivated biologists, and nearly all subsequent work on amphibian salt tolerance and adaptation has been written in reference to this, and only one or two other (i.e., *B. viridis*, *Xenopus laevis*) species (Shoemaker et al. 1992). Thus, statements emphasizing these putative model species have remained common to this day, despite evidence that this pattern may be much more widespread. Indeed, most authors introduce their findings of salt tolerance in their study species by writing something to the effect of: "salt tolerance is extremely rare in amphibians, and until the present study, has only been documented in the Crab-eating Frog and the Green Toad."

The perception that salt tolerance exists only in a few amphibian species has long persisted in the scientific community (with a few exceptions, e.g. Balinsky (1981), Neill (1958)) and has perhaps biased its members in prematurely discounting the presence of amphibians in certain habitats. Herpetologist Edward H. Taylor, in describing "a new ambystomatid salamander adapted to brackish water" (*Ambystoma subsalsum* (= *taylori*)) (Taylor 1943:152), provides a typical example:

> "Dr. Hobart Smith and I visited Lake Alchichica in 1932, but because of the salinity of the water we made no effort to collect salamanders,

> presuming that they could not occur. In 1939 Mr. Dyfrig McH. Forbes, unaware that salt water is usually not tolerated by amphibians, investigated the lake and succeeded in obtaining two ambystomid larvae."

Gadow (1901) stated that "Common salt is poison to the Amphibia," and there is no doubt that amphibians are indeed osmotically challenged organisms, due to their permeable skin and eggs (Shoemaker and Nagy 1977). A plethora of studies have found that salt can lead to increased mortality, developmental deformities, physiological stress, and the alteration of growth and development at (e.g., Ely 1944; Ruibal 1959; Beebee 1985; Padhye and Ghate 1992; Viertel 1999; Turtle 2000; Chinathamby et al. 2006; Dougherty and Smith 2006; Collins and Russell 2009; Karraker and Ruthig 2009; Langhans 2009; Chambers 2011; Duff 2011; Harless et al. 2011; Alexander et al. 2012; Hopkins et al. 2013a,b; Hua and Pierce 2013), and across (i.e., carry-over effects) (Petranka and Doyle 2010; Wu et al. 2012; Hopkins et al. 2014), different life-history stages. This general intolerance has been demonstrated repeatedly (and as such, will not be a focus of this review), and, perhaps as a result, there are no truly marine or saline-specialist amphibian species. Still, the mere presence of so many species of amphibians inhabiting salt-water areas around the world suggests that these animals may be a lot more adaptable than has been suggested for over a century.

Our review challenges the perception of widespread salt intolerance in amphibians by attempting to compile all documented evidence (including a re-examination of Neill, 1958) of these animals inhabiting brackish and saline environments, whether coastal, inland, natural, or anthropogenically altered. This comes

at a critical incipient time, as the biological community begins to become more fully aware of the ability of amphibians to survive in these habitats around the world. Indeed, almost half (44%) of the references in this review describing amphibians in saline habitats, or their tolerances of salt, were published since 2000, and in the last year and a half alone (January 2013 – October 2014), 20 additional species have been described as inhabiting brackish and saline habitats. With so much burgeoning interest in this topic, it is worth stepping back and analyzing our current state of knowledge on the topic. In addition, while there appears to be much recent interest in documenting the occurrence of amphibians in these habitats, we still know very little regarding how adaptations allowing amphibians to live in these habitats might evolve. We thus conclude this review by outlining an evolutionary model of understanding amphibian adaptation to saline environments. Such studies will be important as freshwater resources become increasingly saline in a world of rising sea-levels (Gornitz 1995; Nicholls et al. 1999; Purcell et al. 2008; Rios-López 2008), road deicing salt application (Environment Canada 2001; Thunqvist 2004; Kaushal et al. 2005; Cañedo-Argülles et al. 2013), and secondary salinization (Williams 2001; Christy and Dickman 2002; Chinathamby et al. 2006; Kearney et al. 2012), and we attempt to understand the ability of vulnerable groups such as amphibians to adapt and survive in these habitats.

MATERIALS AND METHODS

Review of the Literature

We reviewed the scientific literature for reports of amphibians inhabiting brackish or saline environments. Pre-1950s, we relied heavily (but not exclusively) on Neill's (1958) compilation. In doing so, we tried to locate the studies referenced, verify that they met our criteria for inclusion, and classified each study into specific categories (see below). Unlike Neill (1958), we did not include reports of amphibians that were found dead or sickly in saline habitats (e.g., Carl 1949; Neill 1958 personal observation), or second-hand accounts of frog calling, for example, in areas that "might have been brackish," or "near [but not on] a beach." (personal observations and Bellairs and Shute (1954) cited in Neill 1958). We included Neill's personal observations but did not include unverified second-hand accounts in Neill's paper, unless the species in question had also been described in a saline habitat in another publication. Post-1950s, we relied heavily on internet searches for scholarly works involving amphibians and saline habitats and included published accounts from natural history surveys, studies of local adaptation or salinity tolerance, natural history notes, or books. While there is a multitude of studies on amphibian osmoregulatory physiology and the effects of road deicing salts on amphibian survival, we did not include species whose tolerance had been physiologically tested, but never reported, even anecdotally, in saline habitats in the field (e.g., *Ambystoma gracile*, Alvarado and Dietz 1970a). We did include some physiological studies of species observed by others in saline habitats, even if the authors had not collected their study subjects from these habitats (e.g., *Lithobates catesbeianus*, Alvarado and Moody 1970).

In summary, our criteria for inclusion in this review were that at least one author had found at least one life-history stage of the species alive and healthy in a saline environment, and the account had been published. Species names follow Frost (2014).

Classification of Habitats

Our literature review for amphibians inhabiting saline environments revealed a diversity of habitats. These included habitats naturally influenced by oceanic salt, including beaches, lagoons, salt marshes, mangrove swamps, tidal ponds, pools, streams, estuaries, pools affected by sea spray, oceans and bays. All of these habitats were classified as coastal/natural (C/N) in Table 2.1. Other naturally saline habitats included inland seas, saline lakes and ponds, saline hotsprings, and temporary desert ponds and streams recorded as saline. These were classified as inland/natural (I/N) in Table 2.1. We also included habitats (mostly inland) that are affected by anthropogenic sources of salt, such as road deicing salts or secondary salinization, and classified these as such (i.e., A vs. N). Finally, we listed the geographic location of each occurrence.

Classification of Studies

To clarify our understanding of amphibian salt tolerance, we classified all studies/observations in several ways. We recorded (Yes/No) in Table 2.1 whether the study measured the salinity of the reportedly saline environment in which the amphibian was found. Conservatively, those studies that simply reported that the water was "definitely brackish," but did not report salinity measurements (e.g., Peterson et al. 1952)

were not scored as having measured environmental salinity. We also listed whether the authors made a field observation of the animal in a saline environment (Yes/No), and if they subsequently tested salinity tolerance (typically in the laboratory; Yes/No). We also noted those studies that measured some additional aspect of physiological adaptation to salt in the laboratory. Finally, we classified papers as either being a full-length article (FA), natural history note (NHN), or thesis/dissertation (TD), and whether the focus of the paper was on salt tolerance (S) or not (NS).

Estimating Environmental and Experimental Salinity Tolerance Limits

For every species where environmental salinity was measured at the time of field observation, we determined the maximum salinity concentration in which the animal was found. There are many measurement units used in the salinity literature, with very little standardization or consistency (e.g., conductivity, specific conductance, mOsm/L, g/L, mg/mL, mequiv/L, specific gravity, ppt (parts per thousand), ppm (parts per million), psu (practical salinity unit)). To facilitate accurate comparison among species and studies, we converted all values into ppt (g/L Cl^-). For those species whose salt tolerance has been experimentally examined in the laboratory, we determined the maximum upper limit of tolerance by arbitrarily defining this as the concentration of salt in which $\geq 50\%$ of individuals survived. For non-lethal measures, we recorded the upper limit as that concentration which first caused a statistically significant negative effect.

TABLE 2.1.—Amphibians reported from saline habitats and the nature of the habitat and study. C = Coastal, I = Inland, N = Natural, A = Anthropogenic; FA = Full Article, NHN = Natural history note, TD = Thesis or Dissertation; S = salinity-focused, NS = Not salinity-focused.

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
GYMNOPHIONA									
TYPHLONECTIDAE									
Atretochoana eiselti	Adult	Tidal stream, Tidal pool (C/N)	Brazil	No	No	Yes	No	FA/NS	(Hoogmoed et al. 2011)
CAUDATA									
AMBYSTOMATIDAE									
Ambystoma maculatum	Adult, Juvenile	Beach, under driftwood (C/N)	USA	No	No	Yes	No	NHN/NS	(Hardy 1952)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Eggs	Roadside pools (I/A)	USA	Yes	Yes	Yes	No	FA/S	(Turtle 2000; Brady 2012)
	Adults, Eggs, Larvae	Roadside pools (I/A)	USA	Yes	Yes	Yes	No	FA/S	(Karraker et al. 2008)
A. opacum	Adults, Larvae	Beach ponds with salt spray (C/N)	USA	No	No	Yes	No	NHN/NS	(Hardy 1972)
A. talpoideum	Adults, Larvae	Coastal wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)
A. taylori	Adult, Larvae	Saline Lake (I/N)	Mexico	Yes	Yes	Yes	No	FA/NS	(Taylor 1943; Brandon et al. 1981)
A. tigrinum	Larvae	Saline, alkaline pond (I/N)	USA	Yes	Yes	Yes	Yes	FA/S	(Gasser and Miller 1986)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Larvae/ Neotenic Adults	Saline lake (I/N)	USA	Yes	No	Yes	No	FA/S / NHN/NS / FA/NS	(Young 1924; Larson 1968; Held and Peterka 1974)
	Larvae/ Neotenic Adults	Saline Lake (I/N)	USA	Yes	No	Yes	Yes	FA/S	(Duerr and Ness 1970)
	Larvae	Saline Lake (I/N)	Canada	Yes	No	Yes	No	NHN/S	(Hammer 1986)
	Larvae		USA	No	Yes	No	Yes	FA/S	(Kirschner et al. 1971)
	Adults		USA	No	Yes	No	Yes	FA/S	(Romspert and McClanahan 1981)
Dicamptodon tenebrosus	Larvae	Tidal Stream (C/N)	USA	No	No	Yes	No	NHN/S	(Ferguson 1956)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Larvae	Tidal Stream (C/N)	USA	Yes	No	Yes	No	NHN/S	(Hopkins and Hopkins in press)
AMPHIUMIDAE									
Amphiuma means	Adults, Larvae	Coastal wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)
PLETHODONTIDAE									
Batrachoseps gavilanensis	Adults	Beach, under driftwood (C/N)	USA	No	Yes	Yes	Yes	FA/S	(Licht 1975)
B. pacificus	Adults	Beach, under driftwood (C/N)	USA	No	No	Yes	No	FA/NS	(Hansen et al. 2005)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
Eurycea quadridigitata	Adults, Larvae	Coastal wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)
SALAMANDRIDAE									
Lissotriton helveticus	Larvae	Brackish Tidal Pool (C/N)	UK	Yes	No	Yes	No	NHN/S	(Spurway 1943)
	Adults	Island Pond with Sea-Spray (C/N)	UK	Yes	No	Yes	No	FA/NS	(Pyefinch 1937)
	Adults	Coastal Saline Wetland (C/N)	France	Yes	No	Yes	No	FA/S	(Thirion 2014)
L. vulgaris	Adults, Larvae, Eggs	Brackish Tidal Pools (C/N)	UK	No	No	Yes	No	NHN/S	(Hardy 1943)
	Adults, Eggs	Baltic Sea (C/N)	Sweden	No	No	Yes	No	NHN/S	(Hagström 1981)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Adults	Saline lake (I/N)	Russia (W. Siberia)	Yes	No	Yes	No	FA/S	(Decksbach 1922)
Notophthalmus viridescens	Adults	Brackish water (I/N)	USA	No	No	Yes	No	NHN/NS	(Pawling 1939)
	Adults		USA	No	No	No	Yes	FA/S	(Wittig and Brown 1977)
	Adults, Larvae	Coastal wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)
Pleurodeles poireti	Adults	Brackish ponds, estuarine marshes (C/N)	Algeria	No	No	Yes	No	FA/NS	(Samraoui et al. 2012)
Salamandra salamandra	Adults	Semi-arid pools (I/N)	Israel	No	Yes	Yes	Yes	FA/S	(Degani 1981)
Taricha granulosa	Adults	Tidal Stream (C/N)	USA	No	No	Yes	No	NHN/S	(Ferguson 1956)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Adults	Tidal Stream (C/N)	USA	Yes	No	Yes	No	NHN/S	(Hopkins and Hopkins in press)
	Eggs, Larvae	Inland pond (I/A)	USA	No	Yes	No	No	FA/S	(Hopkins et al. 2013b, 2014)
Triturus dobrogicus	Neotenic Adult	Saline Soda Pan (I/N)	Hungary	Yes	No	Yes	No	NHN/S	(Mester et al. 2013)
T. marmoratus	Adults	Brackish Marsh (C/N)	France	Yes	No	Yes	No	FA/S	(Thirion 2014)
SIRENIDAE									
Siren lacertina	Adults	Mangrove swamp (C/N)	USA	Yes	No	Yes	No	NHN/S	(Boss and Chesnes 2014)
ANURA									
ALYTIDAE									

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
Discoglossus galganoi	Adults	Brackish water	Spain	No	No	Yes	No	FA/NS	(Nöllert and Nöllert 1992)
D. pictus	Larvae	Coastal saline lake, Brackish lagoon (C/N)	Morocco	Yes	No	Yes	No	FA/NS	(El Hamoumi et al. 2007)
		Salt marshes, estuaries, brackish ponds (C/N)	Tunisia, Algeria, France	Yes	Yes	Yes	No	FA/NS	(Knoepffler 1962)
D. sardus	Larvae	Salt marshes, estuaries, brackish ponds (C/N)	Tunisia, Algeria, France	Yes	Yes	Yes	No	FA/NS	(Knoepffler 1962)
BOMBINATORIDAE									
Bombina	Adults,	Brackish Ditch	France	Yes	No	Yes	No	TD/S	(Florentin 1899)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
variegata	Larvae	(I/N)							
	Larvae	Saline discharges/flows (I/N)	Germany	No	No	Yes	No	FA/NS	(Knoepffler 1962)
BUFONIDAE									
Amietophrynus mauritanicus	Adults, Larvae,	Beach, Stream on Beach (C/N)	Algeria	No	No	Yes	No	NHN/NS	(Bellairs and Shute 1954)
	Adults	Brackish pond (C/N)	Algeria	No	No	Yes	No	FA/NS	(Samraoui et al. 2012)
Anaxyrus americanus	Adults	Tidal Marsh (C/N)	Canada	Yes	No	Yes	No	FA/NS	(Ouellet et al. 2009)
	Adults, Eggs	Tidal Marsh (C/N)	USA	Yes	No	Yes	No	NHN/S	(Kiviat and Stapleton 1983)
	Adults, Eggs,	Roadside wetlands (I/A)	Canada	Yes	Yes	Yes	No	FA/S	(Collins and Russell 2009)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Larvae								
	Larvae	Road deicing salt (I/A)	USA	No	Yes	No	No	FA/S	(Dougherty and Smith 2006)
	Eggs, Larvae	Road deicing salt (I/A)	USA	No	Yes	No	No	FA/S	(Snodgrass et al. 2008)
A. boreas	Adults	Beach, Ocean (C/N)	USA	No	No	Yes	No	FA/NS	(Storer 1925)
	Adults, Larvae	Saline hotspring, Lake (I/N)	USA	Yes	No	Yes	No	FA/NS	(Brues 1932)
	Adults	Saline lake (I/N)	USA	No	No	Yes	No	FA/NS	(Brues 1932)
A. fowleri	Adults	Beach, Beach ponds with salt spray, Coastal Islands, Ocean	USA	No	No	Yes	No	FA/NH N/NS	(Wright and Wright 1938; Engels 1952; Hardy 1972)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		(C/N)							
A. quercicus	Adults, Larvae	Beach, Coastal Islands, Coastal wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/NS	(Engels 1952; Gunzburger et al. 2010)
A. terrestris	Adults, Larvae	Beach, Coastal Islands, Coastal Wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/NS/ S/NHN/ S	(Allen 1932; Smith and List 1955; Neill 1958; Gunzburger et al. 2010)
	Larvae	Inland freshwater	USA	No	Yes	No	No	FA/S	(Brown and Walls 2013)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
Bufo bufo	Adults, Eggs	Brackish Pools (C/N)	UK	No	No	Yes	No	NHN/S	(Hardy 1943)
	Larvae	Freshwater Pond (C/N)	Italy	Yes	Yes	Yes	Yes	FA/S	(Bernabò et al. 2013)
	Larvae	Brackish Island Pool (C/N)	Norway	Yes	No	Yes	No	NHN/S	(Hagström 1981)
	Larvae	Brackish ditch (I/N)	France	Yes	No	Yes	No	TD/S	(Florentin 1899)
Bufotes balearicus	Larvae	Pond (C/N)	Italy	Yes	Yes	Yes	Yes	FA/S	(Bernabò et al. 2013)
B. boulengeri	Larvae	Coastal saline lake, Brackish lagoon (C/N)	Morocco	Yes	No	Yes	No	FA/NS	(El Hamoumi et al. 2007)
	Adults	Brackish water (C/N)	Algeria, Egypt	No	No	Yes	No	FA/NS	(Werner 1909)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
B. variabilis	Adults	Beach (C/N)	Iran	No	No	Yes	No	FA/NS	(Schmidt 1955)
	Adults	Shores of hypersaline lake (I/N)	Iran	No	No	Yes	No	FA/NS	(Asem et al. 2014)
B. viridis		Saline, muddy pools (I/N)	Austria	No	No	Yes	No	FA/NS	(Knoepffler 1962)
	Adults	Brackish pools, Ocean (sound) (C/N)	Sweden	Yes	No	Yes	No	FA/NS	(Gislén and Kauri 1959)
	Adults		Belgium, Yugoslavia, Italy, Israel	No	Yes	No	Yes	FA/S	(Gordon 1962; Tercafs and Schoffeniels 1962; Katz 1973,1975)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Adults, Eggs	Baltic Sea (C/N)	Sweden	No	No	Yes	No	NHN/S	(Mertens 1926; Hagström 1981)
	Adults, Eggs	Brackish water (C/N)	Europe	No	No	Yes	No	FA/NS	(Boulenger 1897–1898)
Duttaphrynus melanostictus	Adult	Brackish Ponds/Estuary (C/N)	India	Yes	No	Yes	No	FA/NS	(Annandale 1907)
	Adult	Saline mangrove swamp (C/N)	Bangladesh	No	No	Yes	No	FA/NS	(Rahman and Asaduzzaman 2010)
	Adult	Pond (C/N)	India	No	Yes	No	Yes	FA/S	(Chakko 1968)
	Larvae	Pond (C/N)	Hong Kong	No	Yes	No	No	FA/S	(Strahan 1957; Karraker et al. 2010)
	Adult	Brackish mangrove	Singapore	No	No	Yes	No	NHN/NS	(Chan and Goh 2010)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		swamp (C/N)							
Epidalea calamita	Adults, Eggs	Brackish pools, tidal pools, estuaries (C/N)	UK	No	No	Yes	No	FA/NS	(Boulenger 1897–1898,1920b; Hardy 1943)
	Adults, Eggs	Baltic sea (C/N)	Europe	No	No	Yes	No	FA/NHN/NS	(Mertens 1926; Hagström 1981)
	Eggs, Larvae	Brackish beach pool (C/N)	UK	Yes	Yes	Yes	No	FA/S	(Beebee 1985)
	Adults, Eggs	Ocean (bay) (C/N)	Sweden	Yes	No	Yes	No	FA/NS	(Gislén and Kauri 1959)
	Larvae	Saline pools on Frisian Islands (C/N)	Germany	No	No	Yes	No	FA/NS	(Knoepffler 1962)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		Saline tidal marsh (C/N)	France	No	No	Yes	No	FA/NS	(Knoepffler 1962)
	Larvae	Coastal Saline Wetlands, Salt Marsh (C/N)	France	Yes	No	Yes	No	FA/S	(Thirion 2014)
	Eggs, Larvae, Juvenile	Brackish Ponds (I/N)	Spain	Yes	Yes	Yes	No	FA/S	(Gomez-Mestre and Tejedo 2003,2004; Gomez-Mestre et al. 2004; Gomez-Mestre and Tejedo 2005)
	Larvae	Brackish Ponds (I/N)	Spain	Yes	Yes	Yes	Yes	FA/S	(Gomez-Mestre et al. 2004)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
Incilius nebulifer	Eggs, Larvae	Ditch (C/N)	USA	No	Yes	No	Yes	FA/S	(Alexander et al. 2012)
I. valliceps	Adults, Eggs	Brackish Coastal Salt Marshes, Wetlands impacted by storm tides (C/N)	USA	No	No	Yes	No	FA/NS	(Burger et al. 1949; Neill 1958; Mueller 1985)
Peltophryne lemur	Adults, eggs	Brackish Pools (C/N)	Puerto Rico	Yes	No	Yes	No	TD/NS	(Matos-Torres 2006)
	Adults	Mangrove swamps (C/N)	British Virgin Islands	No	No	Yes	No	FA/NS	(Grant 1932)
Rhinella arenarum	Adults, Larvae	Brackish salt flats stream	Argentina	Yes	Yes	Yes	Yes	FA/S	(Ruibal 1962)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		(I/N)							
R. crucifer	Larvae	Brackish Estuary (C/N)	Brazil	Yes	No	Yes	No	NHN/S	(Guix and Lopes 1989)
R. dorbignyi	Adults, Eggs	Coastal lagoon with artificially opened sand-bar (C/N/A)	Brazil	Yes	No	Yes	No	FA/S	(Moreira et al. 2014)
R. marina	Adults	Brackish pools, Beach, Mangroves (C/N)	Australia	No	No	Yes	No	FA/NS	(van Beurden and Grigg 1980)
	Adults		Australia	No	Yes	No	Yes	FA/S	(Liggins and Grigg 1985)
	Eggs, Larvae		USA (Hawaii)	No	Yes	No	No	NHN/S	(Ely 1944)
	Adults,	Temporal pools	Costa Rica	No	No	Yes	No	FA/NS	(Sasa et al.

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Eggs, Larvae	on beach (C/N)							2009)
	Adults, Larvae	Saline mangroves, Brackish swamp/forest (C/N)	Puerto Rico	Yes	Yes	Yes	No	FA/S	(Rios-López 2008)
Strauchbufo raddei	Adult	Beach, Ocean (C/N)	China	No	No	Yes	No	NHN/S	(Shaw 1934)
CERATOPHRYIDAE									
Chacophrys pierottii	Adults	Brackish salt flats pools (I/N)	Argentina	No	No	Yes	No	FA/NS	(Cei 1955)
Lepidobatrachus asper	Adults	Brackish salt flats pools (I/N)	Argentina	Yes	Yes	Yes	Yes	FA/S	(Ruibal 1962)
CRAUGASTORIDAE									

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
Craugastor fitzingeri	Adults	Beach (C/N)	Costa Rica	No	No	Yes	No	FA/NS	(Sasa et al. 2009)
CYCLORAMPHIDAE									
Thoropa taophora	Adults, Larvae	Intertidal zone of seashore (C/N)	Brazil	No	No	Yes	No	FA/NS	(Sazima 1971; Brasileiro et al. 2010)
	Adults	Intertidal zone of seashore (C/N)	Brazil	No	No	Yes	Yes	FA/S	(Abe and Bicudo 1991)
	Adults, Larvae	Rocky beach (C/N)	Brazil	No	No	Yes	No	FA/NS	(Muralidhar et al. 2014)
DENDROBATIDAE									
Hyloxalus littoralis	Adults	Pond on beach (C/N)	Peru	No	No	Yes	No	FA/NS	(Péfaur 1984)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
DICROGLOSSIDAE									
Euphlyctis cyanophlyctis	Adult	Brackish Ponds/Estuary (C/N)	India	Yes	No	Yes	No	FA/NS	(Annandale 1907)
	Adult	Pond (C/N)	India	No	Yes	No	Yes	FA/S	(Chakko 1968)
	Adults	Tidal Mangrove swamp (C/N)	India, Bangladesh	No	No	Yes	No	FA/NS	(Rahman and Asaduzzaman 2010; Jena et al. 2013)
E. hexadactylus	Adults	Tidal Mangrove swamp (C/N)	India	No	No	Yes	No	FA/NS	(Jena et al. 2013)
Fejervarya cancrivora	Adults	Tidal Mangrove swamp (C/N)	India	No	No	Yes	No	FA/NS	(Satheeshkumar 2011; Jena et al. 2013)
	Adults	Tidal stream,	Myanmar	No	No	Yes	No	FA/NS	(Wogan et al.

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		mangrove forest (C/N)							2008)
	Adults	Beach, Ocean (C/N)	South Asia	No	No	Yes	No	FA/NS	(Boulenger 1920a)
	Adults	Brackish Pools (C/N)	Philippines	No	No	Yes	No	FA/NS	(Alcala 1962)
	Adults	Brackish water mangrove swamps (C/N)	Singapore	No	No	Yes	No	NHN/NS	(Chan and Goh 2010)
	Larvae	Intertidal zone on Beach (crab burrows) (C/N)	Philippines	Yes	No	Yes	No	NHN/S	(Pearse 1911)
	Larvae	Mangrove tidal pools (C/N)	Philippines	Yes	Yes	Yes	No	NHN/S	(Dunson 1977)
	Adults, Eggs,	Mangrove swamps (C/N)	Thailand	Yes	Yes	Yes	Yes	FA/S	(Uchiyama et al. 1990)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Larvae								
	Larvae	Mangrove tidal pools (C/N)	Thailand	Yes	Yes	Yes	Yes	FA/S	(Gordon and Tucker 1965)
	Adults	Mangrove swamps (C/N)	Thailand	Yes	Yes	Yes	Yes	FA/S	(Gordon et al. 1961; Gordon and Tucker 1968)
	Adults	Brackish water at Estuary mouth (C/N)	Thailand	No	No	Yes	No	FA/NS	(Smith 1927)
	Adults	Mangrove tidal pools (C/N)	Indonesia	Yes	Yes	Yes	Yes	FA/S	(Wygoda et al. 2011)
F. limnocharis	Adult	Brackish Tidal streams (C/N)	Southeast Asia	No	No	Yes	No	FA/NS	(Boulenger 1912)
	Larvae	Brackish island tide pools	Taiwan	Yes	Yes	Yes	Yes	FA/S	(Wu and Kam 2009; Wu et al.

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		(C/N)							2012)
	Larvae	Ponds (C/N)	Hong Kong	No	Yes	No	No	FA/S	(Karraker et al. 2010)
	Larvae	Freshwater ditch (C/N)	Thailand	Yes	Yes	No	Yes	FA/S	(Gordon and Tucker 1965)
F. moodiei	Adults, Larvae	Beach, Crab burrows in intertidal zone, Brackish swamps (C/N)	Philippines	No	No	Yes	No	FA/S/NS	(Taylor 1943; Brown et al. 2013)
F. orissaensis	Adults	Tidal Mangrove swamp (C/N)	India	No	No	Yes	No	FA/NS	(Jena et al. 2013)
Hoplobatrachus crassus	Adults	Tidal Mangrove swamp (C/N)	India	No	No	Yes	No	FA/NS	(Jena et al. 2013)
H. rugulosus	Adults	Tidal Irrigation Ditches (C/N)	Malaysia	Yes	Yes	Yes	No	FA/S	(Davenport and Huat 1997)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
H. tigerinus	Adult	Brackish Ponds/Estuary (C/N)	India	Yes	No	Yes	No	FA/NS	(Annandale 1907)
	Adult		Vietnam	No	Yes	No	Yes	FA/S	(Gordon et al. 1961)
	Adults	Tidal Mangrove swamp (C/N)	India	No	No	Yes	No	FA/NS	(Jena et al. 2013)
Zakerana syhadrensis	Adults	Tidal Mangrove swamp (C/N)	India	No	No	Yes	No	FA/NS	(Jena et al. 2013)
ELEUTHERODACTYLIDAE									
Eleutherodactylus caribe	Adults	Coastal Mangroves (C/N)	Haiti	No	No	Yes	No	FA/NS	(Hedges and Thomas 1992)
E. coqui	Adults	Brackish swamp/forest (C/N)	Puerto Rico	Yes	No	Yes	No	FA/S	(Rios-López 2008)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
E. jamaicensis	Adults	Supratidal area of Beach, under coconut husks (C/N)	Jamaica	No	No	Yes	No	NHN/NS	(Grant 1939)
E. luteolus	Adults	Beach (C/N)	Jamaica	No	No	Yes	No	FA/NS	(Goin 1953)
E. martinicensis	Adults	Beach (C/N)	Antigua	No	No	Yes	No	NHN/NS	(Lynn 1957)
E. planirostris	Adults	Stones/Beach at edge of Ocean (C/N)	USA	No	No	Yes	No	NHN/S	(Neill 1958)
HYLIDAE									
Acris crepitans	Adults, Larvae	Beach ponds with salt spray (C/N)	USA	No	No	Yes	No	NHN/NS	(Hardy 1972)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
A. gryllus	Adults, Larvae	Coastal Marsh with Storm Surge, Brackish pools on sand dunes near Ocean (C/N)	USA	Yes	No	Yes	No	FA/NS/S	(Burger et al. 1949; Neill 1958; Gunzburger et al. 2010)
Aparasphenodon bokermanni	Adults	Brackish tidal river (C/N)	Brazil	No	No	Yes	No	FA/NS	(Pombal 1993)
Dendropsophus microcephalus	Adults	Mangroves (C/N)	Colombia	No	No	Yes	No	NHN/NS	(Alvarez-León and De Ayala-Monedero 2000)
Hyla cinerea	Adults	Brackish pools in coastal salt marsh (C/N)	USA	No	No	Yes	No	FA/NS/NHN/S	(Burger et al. 1949; Neill 1958)
	Adults	Ponds subject to salt spray	USA	Yes	No	Yes	No	FA/NS	(Hardy 1953)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		from Chesapeake Bay (C/N)							
	Adults, Eggs	Brackish Pool (C/N)	USA	No	No	Yes	No	NHN/NS	(Peterson et al. 1952)
	Larvae	Bay (C/N)	USA	Yes	No	Yes	No	NHN/S	(Diener 1965)
	Adults, Larvae	Coastal Wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)
	Larvae	Inland freshwater pond (I/N)	USA	No	Yes	No	No	FA/S	(Brown and Walls 2013)
H. femoralis	Adults, Larvae	Coastal wetland with storm	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		surge (C/N)							
H. gratiosa	Adults, Larvae	Coastal wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)
H. meridionalis	Adults	Brackish pond (C/N)	Algeria	No	No	Yes	No	FA/NS	(Samraoui et al. 2012)
	Larvae	Coastal Saline Wetlands, Salt Marshes (C/N)	France	Yes	No	Yes	No	FA/S	(Thirion 2014)
H. sarda	Adults	Brackish ponds (C/N)	Europe	No	No	Yes	No	FA/NS	(Nöllert and Nöllert 1992)
H. savignyi	Adults	Shores of hypersaline lake (I/N)	Iran	No	No	Yes	No	FA/NS	(Asem et al. 2014)
H. versicolor	Adults	Beach, Pools	USA	No	No	Yes	No	FA/NS/	(Viosca 1923;

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		affected by sea spray (C/N)						NHN/S	Neill 1958)
	Larvae	Road deicing salts (I/A)	USA	No	Yes	No	No	FA/S	(Chambers 2011; Van Meter and Swan 2014)
Hypsiboas geographicus	Larvae	Brackish Estuary (C/N)	Brazil	Yes	No	Yes	No	NHN/S	(Guix and Lopes 1989)
H. pulchellus	Adults	Coastal lagoon with artificially opened sand-bar (C/N/A)	Brazil	Yes	No	Yes	No	FA/S	(Moreira et al. 2014)
Litoria aurea	Larvae	Fresh and Brackish (Secondary Salinisation) wetland (I/A)	Australia	Yes	Yes	Yes	No	FA/S	(Christy and Dickman 2002; Kearney et al. 2012)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Adults, Larvae	Brackish estuary (C/N)	Australia	Yes	No	Yes	No	FA/S	(Hamer et al. 2002)
	Adults, Larvae	Ponds adjacent to Ocean and Coastal Lagoons (C/N)	Australia	Yes	No	Yes	No	FA/NS	(Pyke et al. 2002; Pyke et al. 2013)
L. caerulea	Adults, Larvae	Pond adjacent to Coastal Lagoon (C/N)	Australia	Yes	No	Yes	No	FA/NS	(Pyke et al. 2002)
L. cyclorhyncha	Adults, Larvae	Saline Creek (I/N/A)	Australia	Yes	No	Yes	No	NHN/S	(Janicke and Roberts 2010)
L. dentata	Adults, Larvae	Pond adjacent to Coastal Lagoon (C/N)	Australia	Yes	No	Yes	No	FA/NS	(Pyke et al. 2002)
L. peronii	Adults, Larvae	Pond adjacent to Coastal	Australia	Yes	No	Yes	No	FA/NS	(Pyke et al. 2002)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		Lagoon (C/N)							
L. tyleri	Adults, Larvae	Pond adjacent to Coastal Lagoon (C/N)	Australia	Yes	No	Yes	No	FA/NS	(Pyke et al. 2002)
Osteopilus pulchrilineatus	Adults	Coastal Mangroves (C/N)	Haiti	No	No	Yes	No	FA/NS	(Hedges and Thomas 1992)
O. septentrionalis	Adults, Eggs	Brackish Pool (C/N)	USA	No	No	Yes	No	NHN/S	(Peterson et al. 1952; Neill 1958)
	Adults	Mangroves (C/N)	USA	No	No	Yes	No	FA/NS	(Glorioso et al. 2012)
	Larvae	Inland freshwater	USA	No	Yes	No	No	FA/S	(Brown and Walls 2013)
Pseudacris	Adults	Salt marshes	USA	No	No	Yes	No	FA/NS	(Smith and

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
clarkii		very close to ocean (with crabs) (C/N)							Sanders 1952)
P. crucifer	Adults	Tidal marsh (C/N)	Canada	Yes	No	Yes	No	FA/NS	(Ouellet et al. 2009)
	Adults, Eggs, Larvae	Roadside wetlands (I/A)	Canada	Yes	Yes	Yes	No	FA/S	(Collins and Russell 2009)
	Adults, Larvae	Beach ponds with salt spray (C/N)	USA	No	No	Yes	No	NHN/NS	(Hardy 1972)
P. maculata	Adults	Tidal marsh (C/N)	Canada	Yes	No	Yes	No	FA/NS	(Ouellet et al. 2009)
P. nigrita	Adults, Larvae	Coastal wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
P. ocularis	Adults, Larvae	Coastal wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)
P. regilla	Adults	Saline Island Pools (C/N)	USA	No	No	Yes	No	NHN/NS	(Murray 1955)
	Adults, Larvae, Eggs	Beach and cliff Pools in spray zone (near tide mark) (C/N)	USA	Yes	Yes	Yes	Yes	TD/S	(Roberts 1970)
	Adults	Saline hotspring (I/N)	USA	Yes	No	Yes	No	FA/NS	(Brues 1932)
	Adults	Brackish oceanic bay and alkaline marsh (C/N)	USA	Yes	Yes	Yes	Yes	TD/S	(Weick 1980)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Adults, Eggs, Larvae	Brackish marsh, tidal estuary (C/N)	USA	Yes	No	Yes	No	FA/S	(Smith and Reis 1997)
Pseudis paradoxa	Adults, Juveniles	Mangroves, Saline swamp, Tidal river (C/N)	Trinidad	No	No	Yes	No	FA/NS	(Downie et al. 2010)
Scinax ruber	Adults	Mangroves (C/N)	Colombia	No	No	Yes	No	NHN/NS	(Alvarez-León and De Ayala-Monedero 2000)
S. squalirostris	Adults	Coastal lagoon with artificially opened sand-bar (C/N/A)	Brazil	Yes	No	Yes	No	FA/S	(Moreira et al. 2014)
Smilisca baudinii	Adults, Eggs,	Mangroves (C/N)	Costa Rica	No	No	Yes	No	FA/NS	(Sasa et al. 2009)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Larvae								
Trachycephalus typhonius	Adults, Eggs, Larvae	Mangroves (C/N)	Costa Rica	No	No	Yes	No	FA/NS	(Sasa et al. 2009)
LEPTODACTYLIDAE									
Leptodactylus albilabris	Adults, Larvae	Brackish swamp/forest (C/N)	Puerto Rico	Yes	Yes	Yes	No	FA/S	(Rios-López 2008)
L. gracilis	Adults	Coastal lagoon with artificially opened sand-bar (C/N/A)	Brazil	Yes	No	Yes	No	FA/S	(Moreira et al. 2014)
L. latrans	Adults	Crab burrows in mangrove swamps (C/N)	Brazil	No	No	Yes	No	NHN/S	(Ferreira and Tonini 2010)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Adults	Coastal lagoon with artificially opened sand-bar (C/N/A)	Brazil	Yes	No	Yes	No	FA/S	(Moreira et al. 2014)
L. macrosternum	Adults	Mangrove swamp (C/N)	Brazil	Yes	No	Yes	No	NHN/NS	(Andrade et al. 2012)
L. melanonotus	Adults, Eggs, Larvae	Ponds on beach (C/N)	Costa Rica	No	No	Yes	No	FA/NS	(Sasa et al. 2009)
L. nesiotus	Adults	Brackish swamp (C/N)	Trinidad	No	No	Yes	No	FA/NS	(Ponssa et al. 2010)
L. pentadactylus	Adults	Mangrove swamp (C/N)	Guyana	No	No	Yes	No	NHN/NS	(Crawford and Jones 1933)
Physalaemus biligonigerus	Adults	Coastal lagoon with artificially opened sand-	Brazil	Yes	No	Yes	No	FA/S	(Moreira et al. 2014)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		bar (C/N/A)							
P. gracilis	Adults	Coastal lagoon with artificially opened sand-bar (C/N/A)	Brazil	Yes	No	Yes	No	FA/S	(Moreira et al. 2014)
P. henselii	Adults	Coastal lagoon with artificially opened sand-bar (C/N/A)	Brazil	Yes	No	Yes	No	FA/S	(Moreira et al. 2014)
Pleurodema bufoninum	Adults, Eggs	Salt water (C/N)	Argentina	No	No	Yes	No	NHN/NS	(Darwin 1834; Bell 1843)
P. nebulosum	Adults	Brackish salt flats pools (I/N)	Argentina	Yes	Yes	Yes	Yes	FA/S	(Ruibal 1962)
LIMNODYNASTIDAE									

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
Limnodynastes dumerili	Larvae	Saline wetlands / Secondary salinization (I/A)	Australia	Yes	No	Yes	No	FA/S	(Smith et al. 2007)
L. peronii	Larvae	Saline wetlands / Secondary salinization (I/A)	Australia	Yes	No	Yes	No	FA/S	(Smith et al. 2007)
	Adults, Larvae	Pond adjacent to Coastal Lagoon (C/N)	Australia	Yes	No	Yes	No	FA/NS	(Pyke et al. 2002)
L. tasmaniensis	Larvae	Saline wetlands / Secondary salinization (I/A)	Australia	Yes	No	Yes	No	FA/S	(Smith et al. 2007)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
Neobatrachus sudelli	Larvae	Saline wetlands / Secondary salinization (I/A)	Australia	Yes	No	Yes	No	FA/S	(Smith et al. 2007)
MICROHYLIDAE									
Gastrophryne carolinensis	Adults, eggs	Ponds subject to salt spray from Chesapeake Bay (C/N)	USA	Yes	No	Yes	No	FA/NS	(Hardy 1953)
	Adults, eggs	Brackish water Florida Keys (C/N)	USA	No	No	Yes	No	NHN/NS	(Peterson et al. 1952)
	Adults	Beach, Brackish water	USA	No	No	Yes	No	FA/NS	(Viosca 1923; Neill 1958)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		near Beach, Salt Marsh (C/N)							
	Larvae	Inland freshwater	USA	No	Yes	No	No	FA/S	(Brown and Walls 2013)
Glyphoglossus molossus	Adults	Tidal portion of delta (C/N)	Myanmar	No	No	Yes	No	FA/NS	(Theobald 1868)
MYOBATRACHIDAE									
Crinia riparia	Adults	Saline creek (I/N)	Australia	Yes	No	Yes	No	FA/NS	(Odendaal and Bull 1982)
C. signifera	Adults	Saline creek (I/N)	Australia	Yes	No	Yes	No	FA/NS	(Odendaal and Bull 1982)
	Larvae	Brackish tide pools (C/N)	Australia	No	No	Yes	No	FA/NS	(Mokany and Shine 2003)
ODONTOPHRYNIDAE									

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
Odontophrynus maisuma	Adults, Eggs	Coastal lagoon with artificially opened sand-bar (C/N/A)	Brazil	Yes	No	Yes	No	FA/S	(Moreira et al. 2014)
PELOBATIDAE									
Pelobates cultripes	Larvae	Coastal Saline Wetlands, Lagoons, and Salt Marshes (C/N)	France	Yes	No	Yes	No	FA/S	(Thirion 2014)
	Adults	Coastal Wetlands with Tsunami storm surge (C/N)	France	Yes	No	Yes	No	FA/S	(Thirion 2002)
	Eggs	Coastal	France	No	Yes	No	No	TD/NS	(Thirion 2006)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		Wetlands (C/N)							
P. fuscus	Eggs, Larvae	Inland pond polluted with road deicing salts (I/A)	Romania	Yes	Yes	Yes	No	FA/S	(Stanescu et al. 2013)
PELODYTIDAE									
Pelodytes punctatus	Larvae	Coastal Saline Wetlands, Lagoons, and Salt Marshes (C/N)	France	Yes	No	Yes	No	FA/S	(Thirion 2014)
PIPIDAE									
Xenopus laevis	Juveniles	Brackish pond (I/N)	USA	No	Yes	Yes	Yes	FA/S	(Munsey 1972)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Larvae	Road deicing salts (I/A)	USA	No	Yes	No	No	FA/S	(Dougherty and Smith 2006)
	Adults		USA	No	Yes	No	Yes	FA/S	(McBean and Goldstein 1967)
RANIDAE									
Lithobates berlandieri	Adults	Hypersaline lagoon (C/N)	USA	Yes	No	Yes	No	NHN/S	(McCoid 2005)
L. catesbeianus	Adults	Tidal brackish water (C/N)	USA (Hawaii)	No	No	Yes	No	NHN/NS	(La Rivers 1948)
	Adults, Larvae	Beach ponds with salt spray (C/N)	USA	No	No	Yes	No	NHN/NS	(Hardy 1972)
	Eggs, Larvae	Road deicing salt (I/A)	USA	No	Yes	No	No	FA/S	(Matlaga et al. 2014)
	Larvae		USA	No	No	No	Yes	FA/S	(Alvarado and Moody 1970)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Larvae		USA	No	Yes	No	No	FA/S	(Brown and Walls 2013)
L. clamitans	Eggs, Larvae	Road deicing salt (I/A)	USA	No	Yes	No	No	FA/S	(Dougherty and Smith 2006; Karraker 2007)
	Adults, Eggs, Larvae	Roadside wetlands (I/A)	Canada	Yes	Yes	Yes	No	FA/S	(Collins and Russell 2009)
	Adults	Brackish marshes (C/N)	USA	No	No	Yes	No	NHN/S	(Neill 1958)
L. grylio	Adults	Salt marshes (C/N)	USA	No	No	Yes	No	FA/NS/ NHN/S	(Viosca 1923; Neill 1958)
	Adults, Larvae	Coastal Wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Adults	Brackish swamp/forest (C/N)	Puerto Rico	Yes	No	Yes	No	FA/S	(Rios-López 2008)
L. palmipes	Adults	Beach (C/N)	Guyana	No	No	Yes	No	NHN/NS	(Crawford and Jones 1933)
L. pipiens	Adults	Saline Lake (I/N)	USA	Yes	No	Yes	No	FA/NS	(Young 1924)
	Adults	Tidal marsh (C/N)	USA	No	No	Yes	No	FA/NS	(Klemens et al. 1987)
L. sphenocephalus	Adults	Salt marshes (C/N)	USA	Yes	Yes	Yes	Yes	FA/S	(Christman 1974)
	Adults	Salt marshes, intertidal zone, bay, mangrove swamps (C/N)	USA	No	No	Yes	No	NHN/S	(Neill 1958)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Adult, Larvae	Coastal Wetland with storm surge (C/N)	USA	Yes	No	Yes	No	FA/S	(Gunzburger et al. 2010)
	Adults	Brackish bay (C/N)	USA	No	No	Yes	No	FA/NS	(Duellman and Schwartz 1958)
	Larvae		USA	No	Yes	No	No	FA/S	(Brown and Walls 2013)
L. sylvaticus	Adults	Tidal Marsh (C/N)	Canada	Yes	No	Yes	No	FA/NS	(Ouellet et al. 2009)
	Adults, Eggs, Larvae	Road-side wetlands with deicing salt (I/A)	USA	Yes	Yes	Yes	No	FA/S	(Karraker et al. 2008; Brady 2013)
	Larvae	Road deicing salts (I/A)	USA	No	Yes	No	No	FA/S	(Sanzo 2006; Langhans 2009;

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
									Chambers 2011; Harless et al. 2011)
	Eggs, Larvae	Road deicing salts (I/A)	USA	No	Yes	No	No	FA/S	(Snodgrass et al. 2008; Petranka and Doyle 2010)
L. yavapaiensis	Adults, Eggs	Saline creek (connects to Salton Sea) (I/N)	USA	Yes	Yes	Yes	No	FA/S	(Ruibal 1959)
Pelophylax perezi	Eggs	Saline lake (I/N)	Spain	Yes	Yes	Yes	No	FA/S	(Ortiz-Santaliestra et al. 2010)
	Adults	Saline waters (I/N)	Spain	Yes	No	Yes	No	FA/S	(Margalef 1956)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
	Larvae	Coastal Saline Wetlands, Salt Marshes (C/N)	France	Yes	No	Yes	No	FA/S	(Thirion 2014)
	Adults, Eggs, Larvae	Tide Pools (C/N)	Portugal	Yes	No	Yes	No	NHN/S	(Sillero and Ribeiro 2010)
P. ridibundus	Adults	Baltic sea (C/N)	Europe	No	No	Yes	No	FA/NS	(Mertens 1926)
	Adults	Coastal Dune Pond (C/N)	France	Yes	No	Yes	No	FA/S	(Thirion 2014)
	Adults	Saline water (I/N)	Germany	Yes	No	Yes	No	FA/S	(Thienemann 1926)
	Adults	Arid (I/N)	Israel	No	Yes	No	Yes	FA/S	(Katz 1975)
	Adults	Shores of hypersaline	Iran	No	No	Yes	No	FA/NS	(Asem et al. 2014)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
		lake (I/N)							
	Adults	Saline lake (I/N)	Algeria	Yes	No	Yes	No	FA/NS	(Beadle 1943)
P. saharicus	Adults	Brackish pond (C/N), Brackish marsh (I/N)	Algeria	No	No	Yes	No	FA/NS	(Samraoui et al. 2012)
	Adults, Eggs, Larvae	Saline water (I/N)	Algeria	Yes	No	Yes	No	TD/S	(Florentin 1899)
Rana draytonii	Adults, Eggs, Larvae	Brackish marsh, Tidal Estuary (C/N)	USA	Yes	No	Yes	No	TD/S / FA/S	(Smith and Reis 1997; Reis 1999)
R. luteiventris	Adults	Saline hotsprings and Mudflats (I/N)	USA	Yes	No	Yes	No	FA/NS	(Brues 1932; Hovingh 1993)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
R. macrocnemis	Adults	Brackish Coastal and Desert aquatic habitats (C/I/N)	Iran	No	No	Yes	No	FA/NS	(Bahmani et al. 2014)
R. temporaria	Adults, Larvae, Eggs	Brackish Tidal Pools (C/N)	UK	No	No	Yes	No	NHN/S	(Hardy 1943)
	Adults	Baltic sea (C/N)	Europe	No	No	Yes	No	FA/NS	(Mertens 1926)
	Eggs	Inland ponds (I/N)	Germany	Yes	Yes	Yes	No	FA/S	(Viertel 1999)
	Eggs	Brackish ditch (I/N)	France	Yes	No	Yes	No	TD/S	(Florentin 1899)
RHACOPHORIDAE									
Buergeria	Adults,	Beach, Tidal	Japan	No	No	Yes	No	FA/NS	(Goris and

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
japonica	Eggs	streams (C/N)							Maeda 2005)
	Adults, Eggs	Beach, Tidal streams (C/N)	Japan	Yes	No	Yes	No	FA/S	(Haramura 2004,2011)
	Eggs	Beach, Tidal streams (C/N)	Japan	No	Yes	Yes	No	FA/S	(Haramura 2007b)
	Larvae	Tidal stream (C/N)	Japan	Yes	No	Yes	No	FA/S	(Haramura 2007a)
	Adults, Eggs		Japan	No	No	Yes	No	FA/S	(Haramura 2008)
Polypedates maculatus	Adults	Tidal Mangrove swamp (C/N)	India, Bangladesh	No	No	Yes	No	FA/NS	(Rahman and Asaduzzaman 2010; Jena et al. 2013)
P. megacephalus	Larvae	Pond (C/N)	Hong Kong	No	Yes	No	No	FA/S	(Karraker et al. 2010)

Species	Life stage	Habitat	Location	Measured salinity	Tested tolerance	Field observation	Lab physiology	Paper type	Reference
RHINODERMATIDAE									
Rhinoderma darwinii	Adult	Beach (C/N)	Chile	No	No	Yes	No	NHN/NS	(Crump 2002)
SCAPHIOPODIDAE									
Spea hammondii	Adults	Saline hotspring (I/N)	USA	Yes	No	Yes	No	FA/NS	(Brues 1932)

RESULTS AND DISCUSSION

Phylogenetic Breadth

We identified a total of 144 amphibian species, from 65 genera and 28 families, as having representative individuals or populations inhabiting saline habitats (Table 2.1). This included representatives from one of the 10 caecilian families (10%), five of the 9 caudate families (56%), and 22 of the 56 anuran families (39%), representing an impressive breadth across the amphibian tree of life (Fig. 2.1). This review adds 103 species to the number (41) recorded by Neill (1958). The majority of species described here are anurans (124 vs. 19 caudates and 1 caecilian), but this is not surprising given the relative diversity of frogs and toads compared to other amphibians (6431 anurans vs. 687 caudates and 200 caecilians) (Frost 2014).

The large cosmopolitan families Hylidae (32 salt-tolerant species), Bufonidae (21 species), and Ranidae (16 species), as well as the Central and South American Leptodactylidae (12 species), and the Asian species of the Dicroglossidae (10 species) dominated the anurans in this review, whereas Salamandridae (8 species) and Ambystomatidae (6 species) made up the majority of salt-tolerant caudates (Fig. 2.1). Only a couple of individual representatives of one species of aquatic caecilian, *Atretochoana eiselti*, were found in a tidal stream and pool in Brazil (Hoogmoed et al. 2011). The only other hint of salt tolerance in caecilians comes from a study (Measey et al. 2007) of *Schistometopum thomense* (Dermophiidae) on oceanic islands off the coast of West Africa. This species is considered endemic to these islands and is a rare example of an amphibian (let alone a caecilian) on a purely oceanic island. The best explanation for

its occurrence on these islands is oceanic transport on vegetation rafts, which would imply a probable tolerance of oceanic salinity (Measey et al. 2007). While these hints of possible salt tolerance in caecilians are certainly suggestive, our understanding of adaptation to salt in this group of little-studied amphibians is clearly still very much in its infancy.

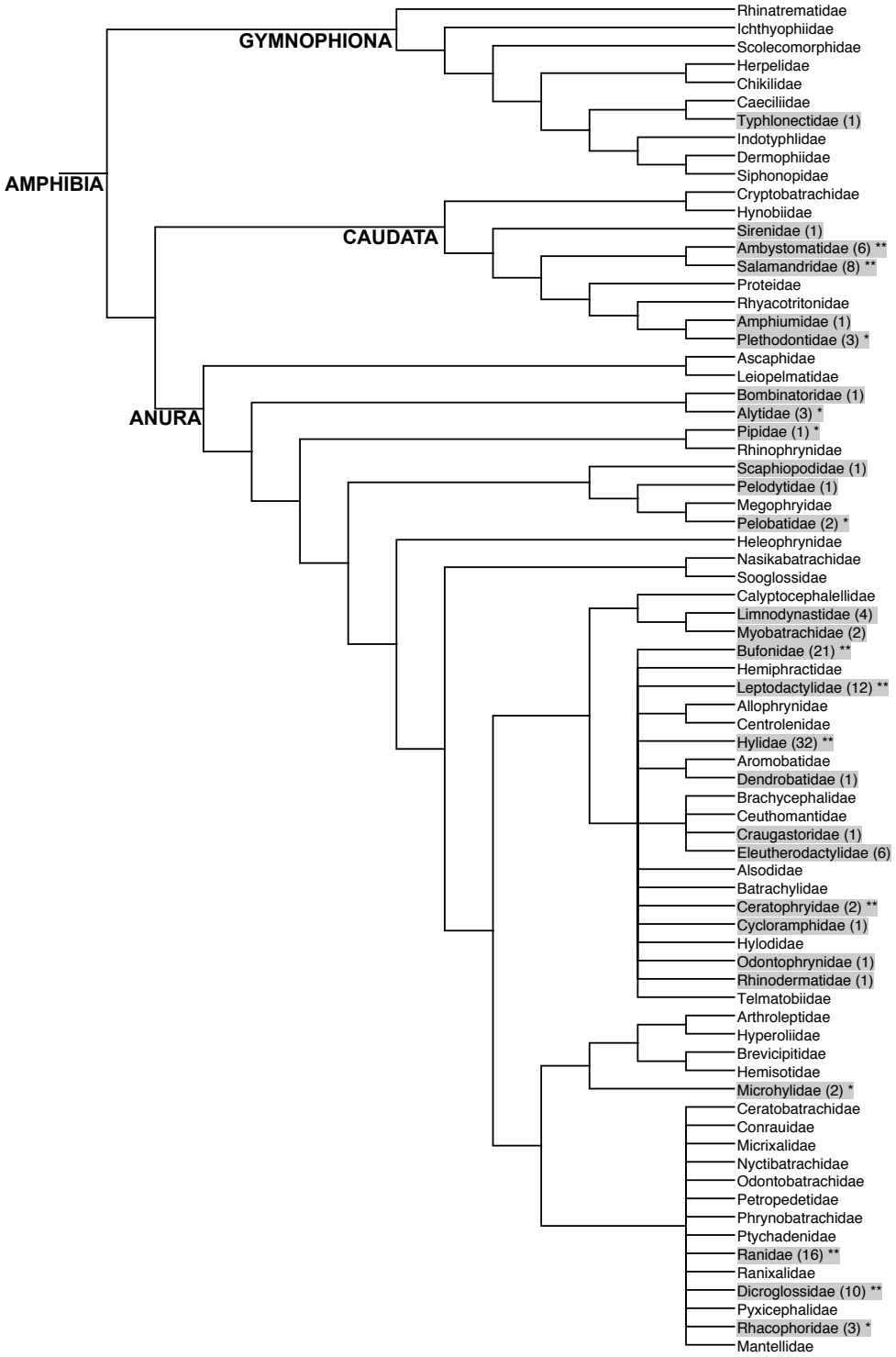

FIG. 2.1.—Phylogeny of amphibian families (Frost, 2014), highlighting those with salt-tolerant representatives. The number of known salt-tolerant species is included in parentheses and families with well-studied species are indicated with asterisks (see Table 2A for **, 2BC for *).

Geographical and Habitat Distribution

Salt-tolerant amphibians have been reported from all continents except Antarctica (i.e., on every continent where amphibians are found), with the majority of species from North America, and, with the exception of Australia, the majority located in naturally saline coastal areas (Fig. 2.2,2.3). A larger count from North America may have more to do with a bias in the number of researchers from this region studying this topic, rather than a biological phenomenon, especially given that the majority of the world's anuran species are found in the tropics rather than North America. Regardless, it seems that wherever amphibians occur, there are examples of salt tolerance having evolved, often in both coastal and inland habitats (Fig. 2.2,2.3).

Although most studies of adaptation to saline habitats have been conducted on natural systems (~95%), a few (e.g., Christy and Dickman 2002; Karraker 2007; Janicke and Roberts 2010; Brady 2012; Kearney et al. 2012; Hopkins et al. 2013b) highlight the importance of examining adaptation in response to anthropogenic sources of salt, principally secondary salinization due to landscape modification and agricultural run-off in Australia, and road deicing salt application in North America (e.g., Fig. 2.3C). While myriad studies have documented the adverse effects of this salinization on amphibians in both habitat types, it appears that some amphibian populations and species have the potential to adapt to artificially elevated levels of salinity in their habitats. This makes sense given amphibians' long evolutionary history of adapting to naturally saline environments, which may give them an edge on adapting to anthropogenic salt (NaCl) over other pollutants (but see later section on "Limitations of Salt Tolerance"). Interestingly, the distinction between natural vs. anthropogenic salinization can also be

blurred, as is the case with salt water intrusion into freshwater bodies due to rising sea levels associated with human-induced climate change (Nicholls et al. 1999). In addition, the influx of seawater into natural coastal estuaries, lagoons and wetlands can also be heavily managed, causing salinities to change dramatically when artificial barriers are purposely breached (Smith and Reis 1997; Moreira et al. 2014). Several species of anurans have been found inhabiting and breeding in these natural/anthropogenic brackish water bodies (Smith and Reis 1997; Moreira et al. 2014), and intriguingly, it is possible that this management technique actually promotes versus discourages amphibian occupancy (Moreira et al. 2014).

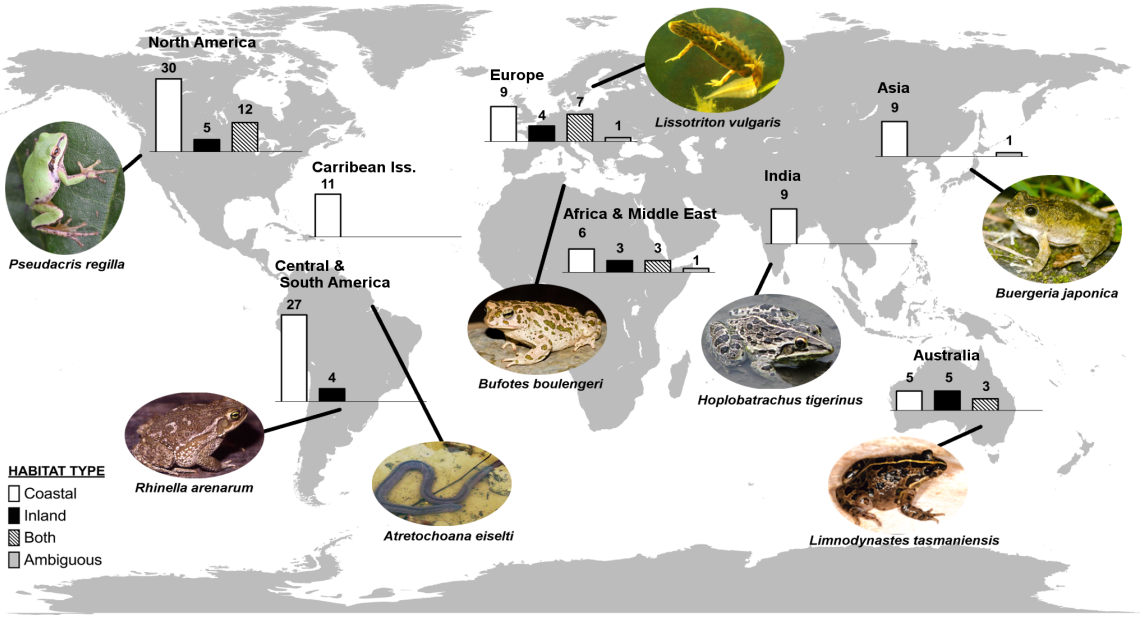

FIG. 2.2.—Global geographic distribution of salt-tolerant amphibian species and the habitat types in which they are found: Coastal = white bars, Inland = black bars, Both coastal and inland = hatched bars, Ambiguous/not listed = gray bars. Numbers of species are indicated above each bar. Photos show representative species found in saline habitats from each continent: *Pseudacris regilla* (photo by: Oregon Department of Fish and Wildlife), *Rhinella arenarum* (photo by: A. Kwet), *Bufotes boulengeri* (photo by: Manuelgvs), *Lissotriton vulgaris* (photo by: Viridiflavus), *Hoplobatrachus tigerinus* (photo by: Balaram Mahalder), *Buergeria japonica* (photo by: Pseudolapiz), *Atretochoana. eiselti* (photo by: M. Hoogmoed), *Limnodynastes tasmaniensis* (photo by: E.D.B.)

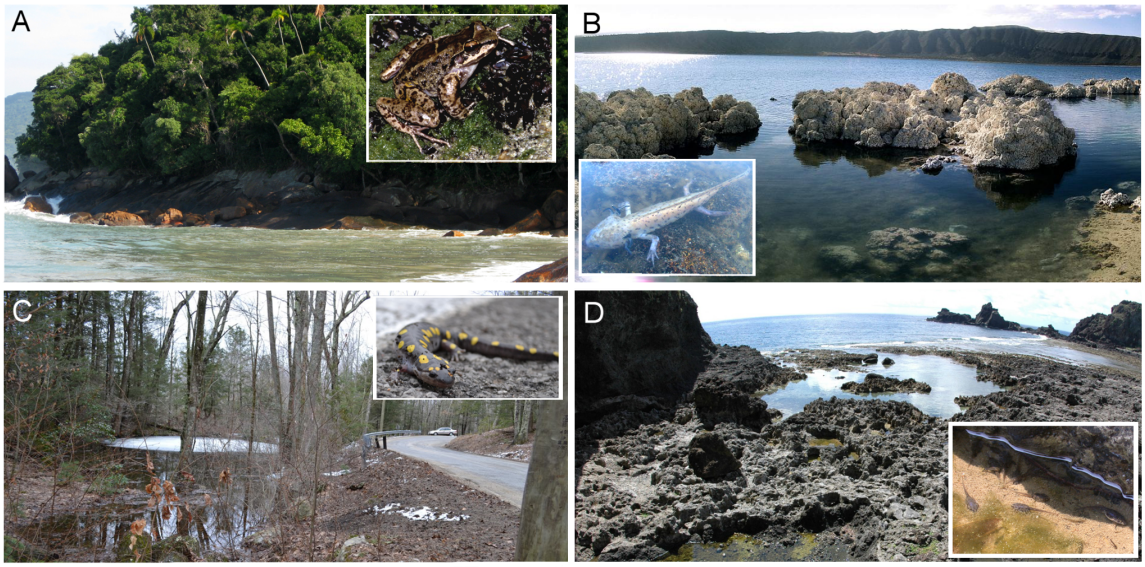

FIG. 2.3.—Examples of different types of saline habitats and their amphibian inhabitants. (A) *Thoropoa taophora* (Cycloramphidae) on a rocky seashore in Brazil (note presence of mussels and barnacles next to frog in inset) (photo by: I. Sazima; see Brasileiro et al. (2010)). (B) Neotenic *Ambystoma taylori* (Ambystomatidae) in inland saline Lake Alchichica, Mexico (photos by: E. De Troya, R. Daniel; see Taylor (1943)). (C) *Ambystoma maculatum* (Ambystomatidae) breeding in a roadside pond salinized by road deicing salts in the eastern USA (photo by: S. Brady; see Brady (2012)). (D) *Fejervarya limnocharis* (Dicroglossidae) tadpoles in tide-pools on islands off the coast of Taiwan (photo by: C.-S. Wu; see Wu and Kam (2009)).

Degree of Understanding

Our data compilation and summary reveal a fairly comprehensive picture of the evolved salt tolerance in at least 42 species of amphibians across 27 genera and 14 families (Table 2.2, Figs. 2.4,2.5). Of these, 17 species (~12% of all studied species; Fig. 2.4, and over half of them from Dicroglossidae or Bufonidae) have been observed in saline habitats in the wild where the environmental salinity of the habitat was measured, the salt tolerance of at least one life-history stage was tested, and some physiological work was performed (Table 2.2A). Another 21 species have been studied in all but their physiology (Table 2.2B), and 4 other species have been found in purportedly saline habitats and their salinity tolerance examined thoroughly (including physiologically), but habitat salinity was not measured (Table 2.2C). The remaining 102 species and their habitats have not been studied in as much detail and reports of their tolerance remain somewhat anecdotal (Fig. 2.4). Some have been tested experimentally for tolerance, and in many cases, environmental salinity was measured. However, in over a quarter of all species, tolerance, environmental salinity, and/or laboratory physiology were not examined (Fig. 2.4). Regardless, their mere presence in putatively saline environments is highly suggestive of salt tolerance. Much more detailed work needs to be done on these species, and undoubtedly many others.

TABLE 2.2.—Well-studied salt-tolerant amphibian species.

A. Species comprehensively studied (environmental salinity, tolerance, field observation, lab physiology)	
Family	Species
Ambystomatidae	*Ambystoma tigrinum*
Bufonidae	*Bufo bufo*
	Bufotes balearicus
	Bufotes viridis
	Duttaphrynus melanostictus
	Epidalea calamita
	Rhinella arenarum
	Rhinella marinus
Ceratophryidae	*Lepidobatrachus asper*
Dicroglossidae	*Euphlyctis cyanophlyctis*
	Fejervarya cancrivora
	Fejervarya limnocharis
	Hoplobatrachus tigerinus
Hylidae	*Pseudacris regilla*
Leptodactylidae	*Pleurodema nebulosum*
Ranidae	*Lithobates sphenocephalus*
	Pelophylax ridibundus
B. Species where all but lab physiology was tested (environmental salinity, tolerance, field observation)	
Ambystomatidae	*Ambystoma maculatum*
	Ambystoma taylori

Salmandridae	*Taricha granulosa*
Alytidae	*Discoglossus pictus*
	Discoglossus sardus
Bufonidae	*Anaxyrus americanus*
	Anaxyrus terrestris
Dicroglossidae	*Hoplobatrachus rugulosus*
Hylidae	*Hyla cinerea*
	Litoria aurea
	Pseudacris crucifer
Leptodactylidae	*Leptodactylus albilabris*
Microhylidae	*Gastrophryne carolinensis*
Pelobatidae	*Pelobates cultripes*
	Pelobates fuscus
Ranidae	*Lithobates clamitans*
	Lithobates sylvaticus
	Lithobates yavapeiensis
	Pelophylax perezi
	Rana temporaria
Rhacophoridae	*Buergeria japonica*

C. Species where all but environmental salinity was tested

(tolerance, field observation, lab physiology)

Plethodontidae	*Batrachoseps gavilanensis*
Salamandridae	*Salamandra salamandra*
Pipidae	*Xenopus laevis*
Ranidae	*Lithobates catesbeianus*

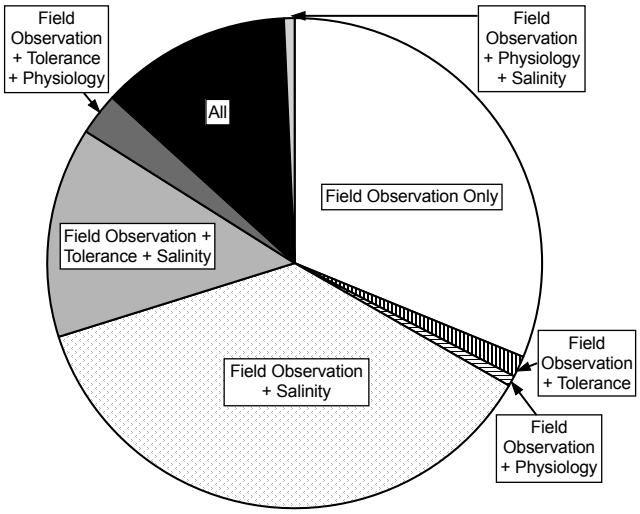

FIG. 2.4.—Proportions of the different aspects of salt tolerance tested in amphibian species. Black corresponds to species listed in Table 2A, Gray to species listed in Table 2.2BC. White are those species that have not been investigated as thoroughly (not in Table 2.2).

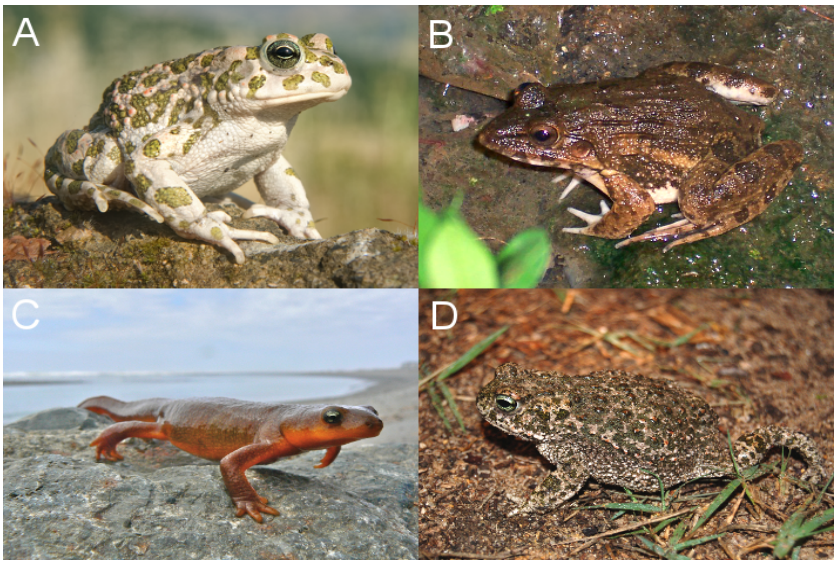

FIG. 2.5.—Examples of well-studied amphibians inhabiting saline habitats (Table 2). (A) *Bufotes balearicus* (= *viridis*) (Bufonidae) in Europe, Africa and the Middle East (photo by: R. Bartz). (B) *Fejervarya cancrivora* (Dicroglossidae) from mangrove swamps in South-east Asia and India (photo by: W.A. Djatmiko). (C) *Taricha granulosa* (Salamandridae) from a tidal stream in North America (photo by: G.R.H.). (D) *Epidalea calamita* (Bufonidae) from a saline dessert pond in Spain (photo by: I. Gomez-Mestre).

Of the 144 species found to inhabit saline habitats, only 24 have had all their life-history stages (eggs, larvae, adults/post-metamorphic juveniles) reported in these habitats or examined for salt tolerance. A total of 131 species have been recorded and/or examined as adults, 75 as larvae, and only 35 as eggs. There is some apparent consensus in the literature that amphibian embryos are most sensitive to salt, followed by larvae, with adults being most tolerant (Gordon et al. 1961; Roberts 1970; Beebee 1985; Padhye and Ghate 1992; Chinathamby et al. 2006; Brand et al. 2010; Petranka and Doyle 2010; Bernabò et al. 2013; Hopkins et al. 2014; Thirion 2014) (although there are also some dissenting data, and evidence that sensitivity can also change with age *within* a particular life stage; see Alexander et al. (2012)). This may be due to differences in the physiological abilities and mechanisms of different life-history stages to regulate salt. Although very little work has been conducted on embryonic physiology, to the best of our knowledge eggs have extremely limited osmoregulatory abilities (Gosner and Black 1957; Karraker and Gibbs 2011), while larvae mainly rely on ionic exchange through gill and integumentary Na^+ pumps (Alvarado and Dietz 1970b; Alvarado and Moody 1970; Gomez-Mestre et al. 2004; Bernabò et al. 2013). Adult amphibians rely both on this integumentary ionic exchange, but also hypersynthesize and retain urea to increase body osmolarity (reviewed by Shoemaker and Nagy 1977; Balinsky 1981; Katz 1989). While some species inhabiting saline habitats appear to avoid egg deposition in highly saline water (e.g., Viertel 1999; Haramura 2008,2011), perhaps due to this apparent sensitivity of eggs, many other species do indeed breed in these habitats, and eggs and larvae have been found in saline waters for numerous species (Table 2.1). More research needs to be conducted on this topic, especially on early life-history stages for which we have a

relative paucity of knowledge, before broad generalizations can be made regarding salt tolerance across life-history stages in amphibians.

Type of Published Work

Over a third of published works were full-length articles with a focus on amphibian adaptation to salinity (Fig. 2.6). Including natural history notes and theses/dissertations, just over half of all articles were focused on salt (Fig. 2.6). This emphasizes the importance of non-salt tolerance literature in reporting on the habits and habitats of amphibians. Many of these articles were general field notes and natural history surveys from the late 1800s – early 1900s, some focused on amphibians (e.g., Boulenger 1897–1898), and others not (e.g., Annandale 1907). More recent articles on faunistic surveys of certain habitats (e.g., Chan and Goh 2010; Jena et al. 2013), range extensions (e.g., Alvarez-León and De Ayala-Monedero 2000; Wogan et al. 2008), and general natural history notes (e.g., Crump 2002) of particular species were equally valuable.

A full review of unpublished dissertations and theses was not completed for this review, but their potential importance to the field is clearly illustrated by the case of *Pseudacris regilla*. Our knowledge of salt tolerance in this species now rivals that of the most well-known salt-tolerant amphibians (Table 2.2A), but only due to the unpublished dissertation of James O. Roberts (1970) and thesis of David L. Weick (1980). These authors found animals in brackish coastal waters, recorded environmental salinity, tested tolerance of locally-adapted populations, and determined the osmoregulatory physiology of animals in these populations. Without these studies, knowledge of salt tolerance in *P.*

regilla would be confined to anecdotal notes (Table 2.1). It is probable that there are many other species of fully-investigated salt-tolerant amphibians residing in the pages of unpublished dissertations and theses that have not made it into this review.

Finally, the importance of natural history notes and short observations, making up just under a quarter of the references in this review (Fig. 2.6), cannot be overstated. These observations were commonplace 100 years ago, but are now published in only a few journals (e.g., Herpetological Review, Herpetology Notes, Herpetological Natural History). They provide valuable insights into a remarkable worldwide phenomenon and may serve as the starting point for more intensive studies. For example, Ferguson's (1956) natural history note of observations of *Taricha granulosa* near the ocean inspired our own studies on *Taricha* salt tolerance (Hopkins et al. 2013b, 2014).

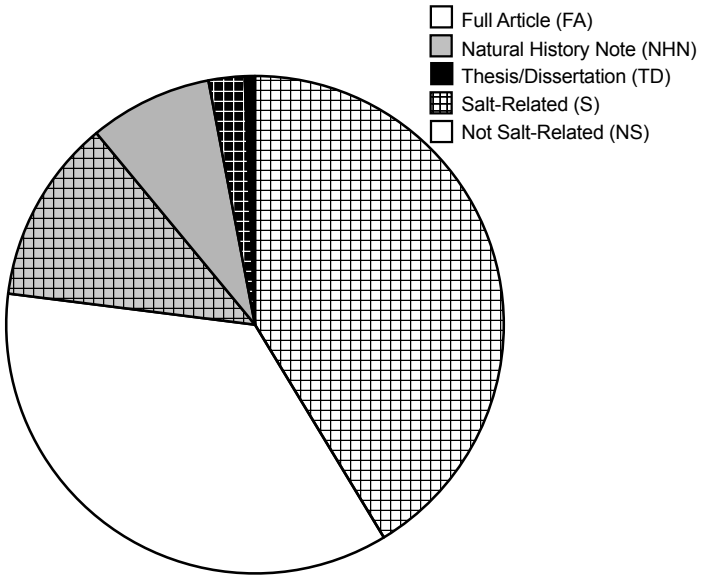

FIG. 2.6.—Classification of the literature as full articles (FA), natural-history notes (NHN), dissertations or theses (TD), and if the work was focused on salt tolerance (S) or not (NS).

Limitations of Salt Tolerance

There may be limits to the concentration of salt to which certain amphibians can adapt. Our review of the literature where environmental salinity was measured and/or salt tolerance was determined experimentally in the laboratory (Table 2.1), indicates that despite amphibians (and especially anurans) being found in, and found to be tolerant of, an extremely wide range of salinities (0.11–39ppt; Table 2.3), the majority of species are found in habitats with maximum salinities of ~2–13ppt, and have a median maximum experimental tolerance of ~9–12ppt (Fig. 2.7). This convergence may therefore represent a general upper limit of salt tolerance for most amphibian species, and was predicted (as 10ppt) by Gomez-Mestre and Tejedo (2003) over 10 years ago. It should be emphasized, however, that this general finding does not necessarily apply to all species, or all populations of a particular species. In particular, we urge caution in directly comparing caudate and anuran species due to the relative paucity of knowledge on caudates, especially regarding experimental tolerance data (only 4 caudate species examined versus 39 anurans; Fig. 2.7). In addition, for this analysis (Table 2.3; Fig. 2.7), we chose the highest salt tolerance level found for a species, not the average among populations. There are certainly many species included here where deleterious effects were seen in individuals from certain populations at lower salinities than were seen in other populations, and where many of the populations were not found in waters as salty as the one population we chose to represent the maximum for this species. In addition, there are clearly several species of anurans that are found in, and can tolerate, extremely high salinities (Table 2.3; Fig. 2.7). Although the most well-known of these euryhaline

amphibians is the Crab-eating Frog (*F. cancrivora*) in salinities up to 39ppt (35ppt measured environmentally) (Gordon et al. 1961; Gordon and Tucker 1968; Dunson 1977; Uchiyama et al. 1990), North America's Rio Grande Leopard Frog (*Lithobates berlandieri*) (McCoid 2005) and Australia's Spotted-thighed Tree Frog (*Litoria cyclorhyncha*) (Janicke and Roberts 2010) have also been found in salinities rivaling or exceeding *F. cancrivora* (39ppt, and 37.4ppt, respectively).

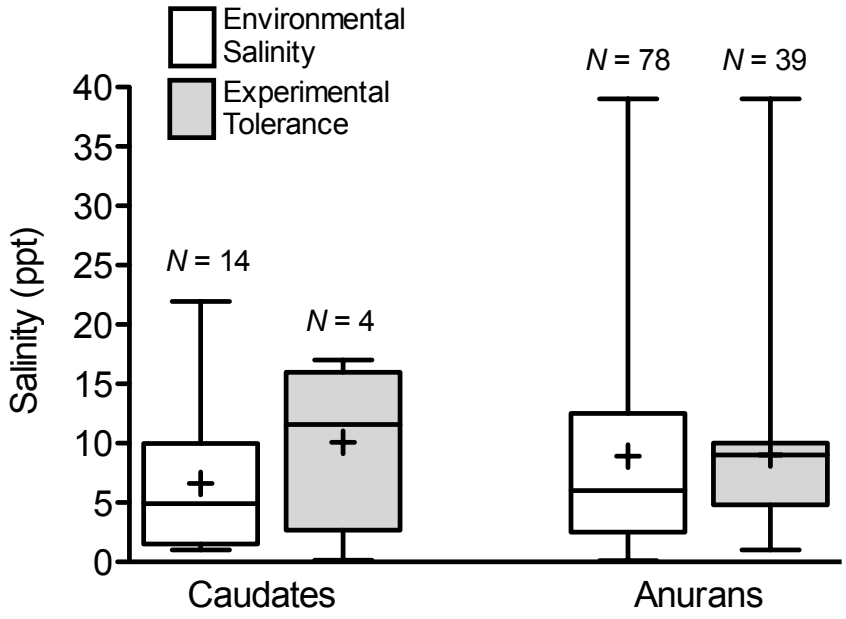

FIG. 2.7.—Maximum salinity concentrations (ppt Cl^-) measured in the field where amphibians were observed (white bars), and maximum salt tolerance limits measured in the lab (grey bars) for caudates and anurans (see Methods text for full definition of tolerance). The range of concentrations is displayed (min to max error bars). Upper and lower box limits represent 3rd (75th percentile) and 1st (25th percentile) quartiles, respectively, with the line in the box representing the median (2nd quartile). Means are shown as plus (+) symbols. *N* numbers indicate the number of species examined.

TABLE 2.3.—Maximum salinity concentrations (ppt Cl^-) measured in the field where amphibians were observed, and maximum salt tolerance limits measured in the lab for amphibian species where these were measured (see Methods text for full definition of tolerance). The references given are for those maximum values listed here, and do not represent the range of values in which species have been found or have been experimentally found to be tolerant.

Species	Life stage	Environmental salinity (ppt)	Experimental tolerance (ppt)	Reference
CAUDATA				
AMBYSTOMATIDAE				
Ambystoma maculatum	Eggs, Larvae	1.56	0.145	(Karraker et al. 2008)
Ambystoma talpoideum	Adults, Larvae	4.9		(Gunzburger et al. 2010)
Ambystoma taylori	Larvae	8.3		(Taylor 1943)
Ambystoma tigrinum	Larvae		10.29	(Gasser and Miller 1986)
	Larvae	15		(Duerr and Ness 1970)
Dicamptodon tenebrosus	Larvae	1		(Hopkins and Hopkins in press)
AMPHIUMIDAE				
Amphiuma means	Adults, Larvae	4.9		(Gunzburger et al. 2010)
SALAMANDRIDAE				
Lissotriton helveticus	Larvae	21.95		(Spurway 1943)
Lissotriton vulgaris	Adults	17		(Decksbach 1922)
Notophlamus viridescens	Adults, Larve	4.9		(Gunzburger et al. 2010)
Salamandra salamandra	Adults		12.9	(Degani 1981)
Taricha granulosa	Adults	1.4		(Hopkins and Hopkins in press)
Triturus dobrogicus	Neotenic Adult	1.72		(Mester et al. 2013)
Triturus marmoratus	Adults	1		(Thirion 2014)
PLETHODONTIDAE				
Batrachoseps gavilanensis	Adult		17	(Licht 1975)
Eurycea quadridigitata	Adults, Larvae	4.9		(Gunzburger et al. 2010)
SIRENIDAE				
Siren lacertina	Adults	4		(Boss and Chesnes 2014)
ANURA				
ALYTIDAE				
Discoglossus pictus	Larvae	6.08	10	(Knoepffler 1962)
Discoglossus sardus	Larvae	9	13	(Knoepffler 1962)
BOMBINATORIDAE				
Bombina variegata	Adults,	13		(Florentin 1899)

Species	Life stage	Environmental salinity (ppt)	Experimental tolerance (ppt)	Reference
	Larvae			
BUFONIDAE				
Anaxyrus americanus	Adults	2		(Ouellet et al. 2009)
	Larvae		3.9	(Collins and Russell 2009)
Anaxyrus boreas	Adults, Larvae	4.5		(Brues 1932)
Anaxyrus quercicus	Adults, Larvae	4.9		(Gunzburger et al. 2010)
Anaxyrus terrestris	Adults, Larvae	4.9		(Gunzburger et al. 2010)
	Larvae		10	(Brown and Walls 2013)
Bufo bufo	Larvae		4.8	(Bernabò et al. 2013)
	Larvae	8		(Florentin 1899)
Bufotes balearicus	Larvae	0.11	6.4	(Bernabò et al. 2013)
Bufotes boulengeri	Larvae	0.21		(El Hamoumi et al. 2007)
Bufotes viridis	Adults	20		(Gislén and Kauri 1959)
	Adults		25	(Tercafs and Schoffeniels 1962)
Duttaphrynus melanostictus	Adults	12.87		(Annandale 1907)
			11.2	(Chakko 1968)
Epidalea calamita	Eggs, Larvae	22	10	(Gomez-Mestre and Tejedo 2003)
Incilius nebulifer	Eggs, Larvae		4	(Alexander et al. 2012)
Peltophryne lemur	Adults, Eggs	2.16		(Matos-Torres 2006)
Rhinella arenarum	Adults, Larvae	4	10	(Ruibal 1962)
Rhinella crucifer	Larvae	18		(Guix and Lopes 1989)
Rhinella marina	Adults, Larvae	20.5		(Rios-López 2008)
			16	(Liggins and Grigg 1985)
CERATOPHRYIDAE				
Lepidobatrachus asper	Adults, Larvae	4	10	(Ruibal 1962)
DICROGLOSSIDAE				
Euphlyctis cyanophlyctis	Adults	12.87		(Annandale 1907)
			8	(Chakko 1968)
Fejervarya cancrivora	Adults, Larvae	35	39	(Gordon et al. 1961)
Fejervarya limnocharis	Larvae	12		(Wu and Kam 2009)
			9.6	(Gordon and Tucker 1965)

Species	Life stage	Environmental salinity (ppt)	Experimental tolerance (ppt)	Reference
Hoplobatrachus rugulosus	Adults	5	10.2	(Davenport and Huat 1997)
Hoplobatrachus tigerinus	Adults	12.87		(Annandale 1907)
			9	(Gordon et al. 1961)
ELEUTHERODACTYLIDAE				
Eleutherodactylus coqui	Adults	20.5		(Rios-López 2008)
HYLIDAE				
Acris gryllus	Adults, Larvae	4.9		(Gunzburger et al. 2010)
Hyla cinerea	Adults	15		(Hardy 1953)
	Larvae		10	(Brown and Walls 2013)
Hyla femoralis	Adults, Larvae	4.9		(Gunzburger et al. 2010)
Hyla gratiosa	Adults, Larvae	4.9		(Gunzburger et al. 2010)
Hyla meridionalis	Adult, Larvae	9		(Thirion 2014)
Hypsiboas geographicus	Larvae	4.5		(Guix and Lopes 1989)
Hypsiboas pulchellus	Adults	2.5		(Moreira et al. 2014)
Litoria aurea	Larvae	7.3		(Pyke et al. 2002)
			5.6	(Kearney et al. 2012)
Litoria caerulea	Adult, Larvae	6		(Pyke et al. 2002)
Litoria cyclorhyncha	Adults, Larvae	37.4		(Janicke and Roberts 2010)
Litoria dentata	Adult, Larvae	6		(Pyke et al. 2002)
Litoria peronii	Adult, Larvae	6		(Pyke et al. 2002)
Litoria tyleri	Adult, Larvae	6		(Pyke et al. 2002)
Osteopilus septentrionalis	Larvae		12	(Brown and Walls 2013)
Pseudacris crucifer	Adults, Larvae	0.59	2.9	(Collins and Russell 2009)
Pseudacris maculata	Adults	2		(Ouellet et al. 2009)
Pseudacris nigrita	Adults, Larvae	4.9		(Gunzburger et al. 2010)
Pseudacris ocularis	Adults, Larvae	4.9		(Gunzburger et al. 2010)
Pseudacris regilla	Adults, Larvae	7.2		(Smith and Reis 1997)
	Adults, Larvae		9.5	(Roberts 1970)
Scinax squalirostris	Adults	2.5		(Moreira et al. 2014)
LEPTODACTYLIDAE				
Leptodactylus albilabris	Adults, Larvae	20.5	4	(Rios-López 2008)
Leptodactylus gracilis	Adults	2.5		(Moreira et al. 2014)
Leptodactylus	Adults	2.5		(Moreira et al. 2014)

Species	Life stage	Environmental salinity (ppt)	Experimental tolerance (ppt)	Reference
latrans				
Leptodactylus macrosternum	Adults	6.4		(Andrade et al. 2012)
Physalaemus biligonigerus	Adults	2.5		(Moreira et al. 2014)
Physalaemus gracilis	Adults	2.5		(Moreira et al. 2014)
Physalaemus henselii	Adults	2.5		(Moreira et al. 2014)
Pleurodema nebulosum	Adults	8	10	(Ruibal 1962)
LIMNODYNASTIDAE				
Limnodynastes dumerili	Larvae	4		Smith et al 2006
Limnodynastes peronii	Adults, Larvae	6		(Pyke et al. 2002)
Limnodynastes tasmaniensis	Larvae	3.9		(Smith et al. 2007)
Neobatrachus sudelli	Larvae	2.64		(Smith et al. 2007)
MICROHYLIDAE				
Gastrophryne carolinensis	Adults, Eggs	15		(Hardy 1953)
	Larvae		5	(Brown and Walls 2013)
MYOBATRACHIDAE				
Crinia riparia	Adults	1.75		(Odendaal and Bull 1982)
Crinia signifera	Adults	0.85		(Odendaal and Bull 1982)
ODONTOPHRYNIDAE				
Odontophrynus maisuma	Adults, Eggs	2.5		(Moreira et al. 2014)
PELOBATIDAE				
Pelobates cultripes	Adult	35		(Thirion 2014)
	Egg		6	(Thirion 2014)
Pelobates fuscus	Larvae, Eggs	0.6	4	(Stanescu et al. 2013)
PELODYTIDAE				
Pelodytes punctatus	Larvae	9		(Thirion 2014)
PIPIDAE				
Xenopus laevis			14	(Munsey 1972)
RANIDAE				
Lithobates berlandieri	Adults	39		(McCoid 2005)
Lithobates catesbeianus	Larvae		10	(Brown and Walls 2013)
Lithobates clamitans	Adults, Eggs, Larvae	0.59	3.1	(Collins and Russell 2009)
Lithobates grylio	Adults	20.5		(Rios-López 2008)
Lithobates pipiens	Adults	15		(Young 1924)
Lithobates	Adults	12.4	10.8	(Christman 1974)

Species	Life stage	Environmental salinity (ppt)	Experimental tolerance (ppt)	Reference
sphenocephalus				
Lithobates sylvaticus	Adults	2		(Ouellet et al. 2009)
	Larvae		7.5	(Harless et al. 2011)
Lithobates yavapaiensis	Adults, Eggs	9	5	(Ruibal 1959)
Pelophylax perezi	Adults, Larvae	28		(Sillero and Ribeiro 2010)
	Eggs		1	(Ortiz-Santaliestra et al. 2010)
Pelophylax ridibundus	Adults	4		(Beadle 1943)
	Adults		8.8	(Katz 1975)
Pelophylax saharicus	Adults, Larvae, Eggs	11		(Florentin 1899)
Rana draytonii	Adults, Larvae	7.2		(Smith and Reis 1997)
Rana pretiosa	Adults	7.6		(Brues 1932)
Rana temporaria	Eggs	4		(Florentin 1899)
			4.5	(Viertel 1999)
RHACOPHORIDAE				
Buergeria japonica	Adults, Eggs	2		(Haramura 2004,2011)
	Eggs		1	(Haramura 2007b)
Polypedates megacephalus	Larvae		6.6	(Karraker et al. 2010)
SCAPHIOPODIDAE				
Spea hammondii	Adults	7.4		(Brues 1932)

CONCLUSIONS

Salt tolerance has evolved in over 100 amphibian species around the world as populations have adapted to exploit coastal and inland saline habitats. The known number of salt-tolerant or salt-adapted species continues to grow rapidly (i.e., 20 since 2013) as we examine amphibian adaptation to both natural and anthropogenic sources of salt. We now understand salinity tolerance in over a dozen species around the world, to a similar extent as well-known examples such as *Fejervarya cancrivora* and *Bufotes*

viridis. Despite this progress, the vast majority of species and families have still not been examined in any depth, and we know very little about salt tolerance and physiological adaptations in most amphibians. More research is needed, especially on understudied groups (such as caecilians and caudates) and life stages (such as eggs), and in areas outside of North America. With over 7200 amphibian species, the number of known salt-tolerant species (144) remains relatively small. However, it is also evident that, while amphibians are osmotically sensitive, they are not helpless, and many are certainly capable of evolving and adapting to saline habitats around the world. As researchers begin to appreciate this worldwide phenomenon, we anticipate that many more salt-tolerant species and populations will be revealed. We encourage biologists to contact us regarding these findings so this review may be updated in the future.

We have now established that salt tolerance in amphibians is not as rare as previously thought, and many of the proximate physiological mechanisms that these animals use in these challenging environments have been elucidated in detail for some species. However, our understanding of the ultimate question, how amphibian populations evolve to be salt-tolerant, is still in its infancy. Given the number of times tolerance has occurred, phylogenetically (Fig. 2.1) and geographically (Fig. 2.2), and continues to evolve in a rapidly changing world (e.g., Brady 2012), our understanding of variation, selective forces, differential survival, and heritability is still mostly unexplored. The rest of this dissertation addresses this evolutionary approach: How does a salt-naïve population evolve and locally adapt to become a more salt-tolerant population or species?

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CHAPTER 3

INTERFAMILY VARIATION IN AMPHIBIAN EARLY LIFE-HISTORY TRAITS: RAW MATERIAL FOR NATURAL SELECTION?[2]

The embryonic development and time to hatching of eggs can be highly adaptive in some species, and thus under selective pressure. In this study we examined the underlying interfamily variation in hatching timing and embryonic development in a population of an oviparous amphibian, the rough-skinned newt (*Taricha granulosa*). We found significant, high variability in degree of embryonic development and hatching timing among eggs from different females. Patterns of variation were present regardless of temperature. We also could not explain the differences among families by morphological traits of the females or their eggs. This study suggests that the variation necessary for natural selection to act upon is present in the early life-history of this amphibian.

[2] Coauthored by Gareth R. Hopkins, Brian G. Gall, Susannah S. French, and Edmund D. Brodie, Jr. Reprinted from Ecology & Evolution Vol. 2, pages 1637-1643, 2012.

INTRODUCTION

Some of the earliest traits subject to natural selection in an oviparous animal's life are those concerning embryonic development and the timing of the first life-history switch-point, when eggs hatch (reviewed by Warkentin 2011). While timing of hatching is often thought to be highly canalized, this has shown to not always be the case in a large variety of taxa, and changes in hatching timing and rates of development can have great effects on the fitness of these organisms (reviewed by Warkentin 2011). For example, variation in the timing of egg hatching in damselflies may allow some individuals to persist while others die in ephemeral habitats where hydroperiod is in constant flux (De Block et al. 2005), and hatching timing in monogenean parasitic worms which infect swimming fish is crucial to facilitating successful host infection (Whittington & Kearn 2011). In amphibians, red-eyed tree frogs (*Agalychnis callidryas*) hatch early to avoid predation by egg-eating snakes and wasps, but this also results in individuals hatching at a smaller size and developmental stage, and thus being more vulnerable to larval predators (Warkentin 1995,1999,2011). The timing and size at which *Ambystoma opacum* and *A. talpoideum* salamanders hatch influences their survival and interspecific competitive and predatory interactions as larvae (Boone et al. 2002), and larvae of the rough-skinned newt, *Taricha granulosa*, that hatch out smaller and less developed are more likely to die from predatory attacks by dragonfly nymphs (Gall et al. 2011a). Thus, the rate and degree of embryonic development that occurs in the egg capsule, and the timing of hatching from that capsule are clearly highly adaptive traits that can have considerable influence on performance during later life stages. However, while clearly

adaptive, it is still unclear if these early life-history traits are subject to natural selection, as we have little knowledge of how they vary within a population.

Variation, regardless of its cause, is the underlying raw material for natural selection. If that variation is heritable, it can lead to evolutionary change. Therefore, although rarely completely possible to do, when trying to understand the potential for evolution, it is important to try to distinguish between the different potential sources of phenotypic variation (Berven 1982; Travis 1983; Laugen et al. 2005).

Temperature is an abiotic environmental influence that can have profound impacts on the embryonic development of amphibians (Bachmann 1969; Brown 1975; Bradford 1990; Voss 1993). Warmer temperatures cause animals to hatch sooner, but at less developed stages than animals raised in colder temperatures (Brown 1975; Williamson & Bull 1989; Voss 1993). We have little knowledge on how these differing environmental temperatures might impact any underlying variation present within a population. There is also a large body of literature which demonstrates that maternal effects such as female and egg size can greatly influence embryonic development and hatching timing (e.g., Kaplan 1980; Crump 1984; Semlitsch & Gibbons 1990; Semlitsch & Schmiedehausen 1994). However, not all studies on these early life-history traits have accounted for maternal effects, as maternal identity is often unknown (e.g., Thumm & Mahony 2002).

The rough-skinned newt (*Taricha granulosa,* Skilton; Caudata: Salamandridae) (Fig. 3.1) is an amphibian species that is well suited to determining the interfamily variation that may be present in embryonic development and hatching timing. Gravid female newts are easily collected and can be induced to deposit their eggs in the

laboratory. Thus, the maternal source of each egg is known, the environment in which the eggs are raised can be controlled, and some morphological characteristics of the female (e.g. size, weight, egg diameter) that might influence embryonic development and hatching timing can be measured and accounted for.

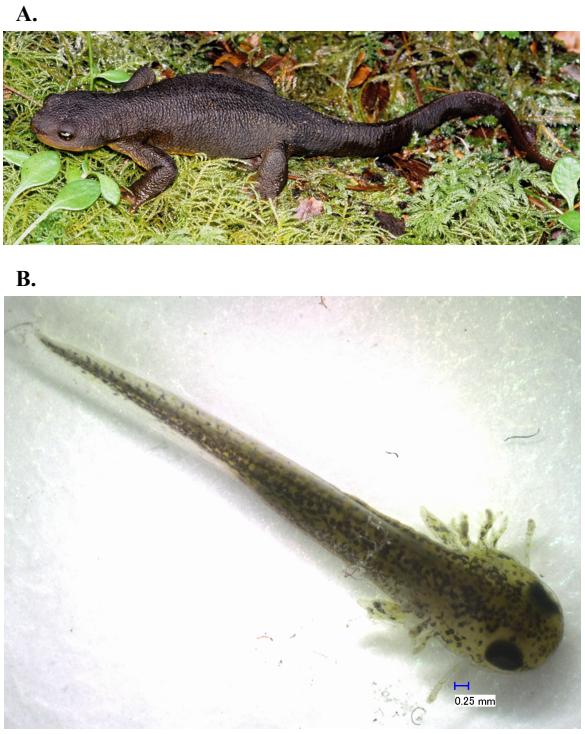

Figure 3.1. The study organism, *Taricha granulosa*. A. Adult newt. B. Newly hatched larva, developmental stage = 42.

The purpose of this study was to determine if the highly adaptive traits of embryonic development and hatching timing varied significantly among the eggs from different *Taricha granulosa* females from a single population (which we hereafter refer to as 'families'). As variation is the basis for natural selection, establishing if this variation

exists is critical for future studies on the evolutionary biology of amphibian early life-history stages. To achieve our goal, we set out to determine if there was underlying variation in hatching timing, developmental stage, and size at hatching among the newt families, and if this variation was present at different environmental temperatures. As the majority of studies on amphibian early life-history traits have not considered differences among families in any of these traits, we used this consensus view in the literature to establish a testable null hypothesis: that there is no variation in hatching timing and embryonic development among newt families from a single population.

MATERIALS AND METHODS

Experimental Animals

A total of 27 gravid, adult female *Taricha granulosa* were collected from Soap Creek ponds in Benton County, Oregon, in 2010 and 2011. These ponds represent a homogenous environment. Animals were transported back to Utah State University and held individually in plastic containers (35 x 20 x 13 cm) with 3.5 l of filtered, chilled tap water. Newts were housed in environmental control chambers at 14 (2010) or 7˚C (2011) and fed blackworms (*Lumbriculus variegatus*) *ad libitum*.

Each female was injected with 10 µl luteinizing hormone releasing hormone ([des-Gly10, D-His(Bzl)6]-LHRH ethylamide; Sigma #L2761) to induce egg deposition and provided a small piece of polyester fiber as an oviposition site. Eggs were collected and removed within 12 hours of deposition, at which point timing of the length of the embryonic period began. After all eggs were deposited, the mass and snout-vent length

(SVL) of each female was recorded. For eggs raised at 7˚C, the mean egg diameter for each female also was calculated by recording the egg diameter for 10 eggs per female (eggs not used in the experiment) using an ocular micrometer with an Olympus stereo microscope.

Embryonic Development at 14˚C

Eggs from 11 female newts were placed into 3.5 cm diameter round numbered cups with 2 ml of filtered tap water in groups of five in March 2010. In total, 444 cups were filled with 2220 eggs. The total number of eggs used from an individual female ranged from 110 to 285.

Cups were placed on shelves in an environmental control chamber at 14˚C and were monitored for hatching at 0700 and 1900 hrs. When a larva had completely hatched and was free-swimming (evident by straightening of the body), the larva was removed from the cup with a pipette, and the time to hatching and developmental stage were recorded. Each larva was photographed (Nikon™ D70 digital camera with a 150 mm micro lens) to determine total length at hatching which was calculated from photos using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland).

We utilized the standard salamander early life-history staging protocol by Harrison (1969), using an Olympus stereo microscope to examine each hatchling for the presence of diagnostic morphologies particular to each stage. This is a 46 stage diagnostic table, and all eggs used at both temperatures hatched between stages 39-43. Stage-specific morphologies include the emergence, length and shape of gills, limb-buds, balancers, eyes, mouth, etc. (see Table 3.1 for more detail).

In this experiment we also exposed developing newt eggs to filtered tap water conditioned with chemical stimuli from one of seven treatments simulating the presence of egg or larval predators; however, chemical stimuli had no effect on the results (main effect or interaction with individual female) and thus will not be discussed further.

Table 3.1. Diagnostic characteristics used to determine developmental stages at hatching in *T. granulosa* hatchlings in this study. Stages encountered include stages 39-43 from Harrison's (1969) 46 stage standard salamander staging table. Descriptions of these stages are reprinted below.

Stage	Description
39	Gills reach forelimb bud, Balancer club-shaped
40	Gills curved dorsally, forelimb bud flattened distally, pigmentation of iris visible
41	Forelimb bud notched distally
42	Forelimb with deeper bifurcation distally and slight bulge marking beginning of elbow joint
43	Mouth opens

Embryonic Development at 7˚C

Eggs from 16 female newts were placed into cups with 2 ml of 20% Holtfreter's solution (a medium recommended for the successful development of caudate embryos (Armstrong et al. 1989)) in groups of 3 in April 2011. Eggs were raised in an environmental control chamber in a similar manner to the 2010 experiment, except at 7˚C instead of 14˚C. In total, 842 cups were filled with 2526 eggs. The total number of eggs used from an individual female ranged from 60 to 294, equating to 20-98 cups (mean ± SE number of cups per female = 52.6 ± 6.3). Cups were checked daily for dehydration, and distilled water was added if needed to maintain a constant water level in each cup.

Cups were checked daily for hatched eggs, at which time the larvae were removed, staged, and their length measured using an ocular micrometer with an Olympus stereo microscope.

Statistical Analyses

We examined the effect of individual female on the hatching time, developmental stage, and total length of recently hatched newt eggs using a general linear model ANOVA with female set as a random factor. Time to hatching, developmental stage, and total length at hatching were analyzed with a normal distribution with the identity link function. All variables met model assumptions of normality and homoscedasticity. Cups were considered replicating units, with eggs within each cup incorporated into the model as subsamples, by using the average value of all eggs within each cup as the unit of analysis.

If the effect of individual female was found to have a significant effect on any of the response variables, we conducted simple linear regressions of female mass and SVL (both temperatures) and mean egg diameter and total number of eggs (7˚C) on the response variables to see if these traits could explain the variation among families.

All statistical analyses were conducted using SAS software Version 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Embryonic Development at 14˚C

There was significant variation (All $P < 0.0001$, Table 3.2) among females in life-history characteristics resulting in some females' offspring hatching more than two days before others (Fig 3.2A), almost a full developmental stage earlier (Fig 3.3A) and 0.8 mm smaller (Fig 3.4A).

There were no biologically meaningful (i.e., strong) relationships between the response variables and female SVL or mass. Although some regressions produced statistically significant results, no R^2 value was greater than 0.07, with the majority being much smaller (mean ± SE $R^2 = 0.02 \pm 0.001$); this indicates that female SVL and mass explained very little of the variation we observed in time to hatching, stage at hatching and length at hatching.

Embryonic Development at 7˚C

Mean time to hatching varied by nearly 7 days (Fig 3.2B), with some families hatching nearly 2 developmental stages earlier (Fig 3.3B) and 1 mm smaller (Fig 3.4B) on average than conspecific families. There was a significant effect of individual female on time to hatching, stage at hatching and size at hatching (all $P < 0.0001$, Table 3.2).

No biologically meaningful relationships were evident between the response variables and female SVL, mass, mean egg diameter, or number of eggs laid. Although some regressions produced statistically significant results, no R^2 value was greater than 0.06, with the majority being much smaller (mean $R^2 = 0.02 \pm 0.004$); this indicates that,

like at 14˚C, any measured female characteristic explained very little of the variation we observed in time to hatching, stage at hatching and length at hatching.

Temperature Effects

Eggs took longer to hatch at 7˚C than at 14˚C (mean = 46.6 days vs. 17.4 days) (Fig 3.2). Hatchlings raised at 7˚C were smaller, but further developed, than hatchlings reared at 14˚C (Figs 3.3 and 3.4). Although interfamily variation was highly significant at both temperatures (all $P < 0.0001$, Table 3.2), the amount of variation at 7˚C appeared to be greater overall than at 14˚C (average range in time to hatching = 2.4 days at 14˚C vs 6.3 days at 7˚C; stage at hatching= 0.87 vs 1.6; size at hatching = 0.77 vs 1.04). There was a significant difference in the variances of the two sets of females for time to hatching ($F_{15,10} = 4.731$, $P = 0.017$) and stage at hatching ($F_{15,10} = 3.941$, $P = 0.034$), but not size at hatching ($F_{15,10} = 2.031$, $P = 0.260$).

Table 3.2. Statistical results of the effect of individual female on time to hatching (days), stage at hatching, and size (length - mm) at hatching for *T. granulosa* embryos raised at 14˚C and 7˚C.

	14˚C						7˚C					
	N	Mean (SD)	*F*	MS (Model, Error)	DF (n,d)	*P*	N	Mean (SD)	*F*	MS (Model, Error)	DF (n,d)	*P*
Time to Hatching	444	17.37 (1.23)	20.97	21.35, 1.02	11, 432	<0.0001	842	49.40 (5.24)	4.92	126.28, 25.68	15, 826	<0.0001
Stage at Hatching	444	40.23 (0.48)	9.98	1.89, 0.19	11, 432	<0.0001	841	41.65 (1.11)	7.37	8.14, 1.10	15, 825	<0.0001
Size at Hatching	444	8.94 (0.40)	21.81	2.13, 0.11	10, 359	<0.0001	842	7.67 (0.72)	12.29	5.26, 0.43	15, 826	<0.0001

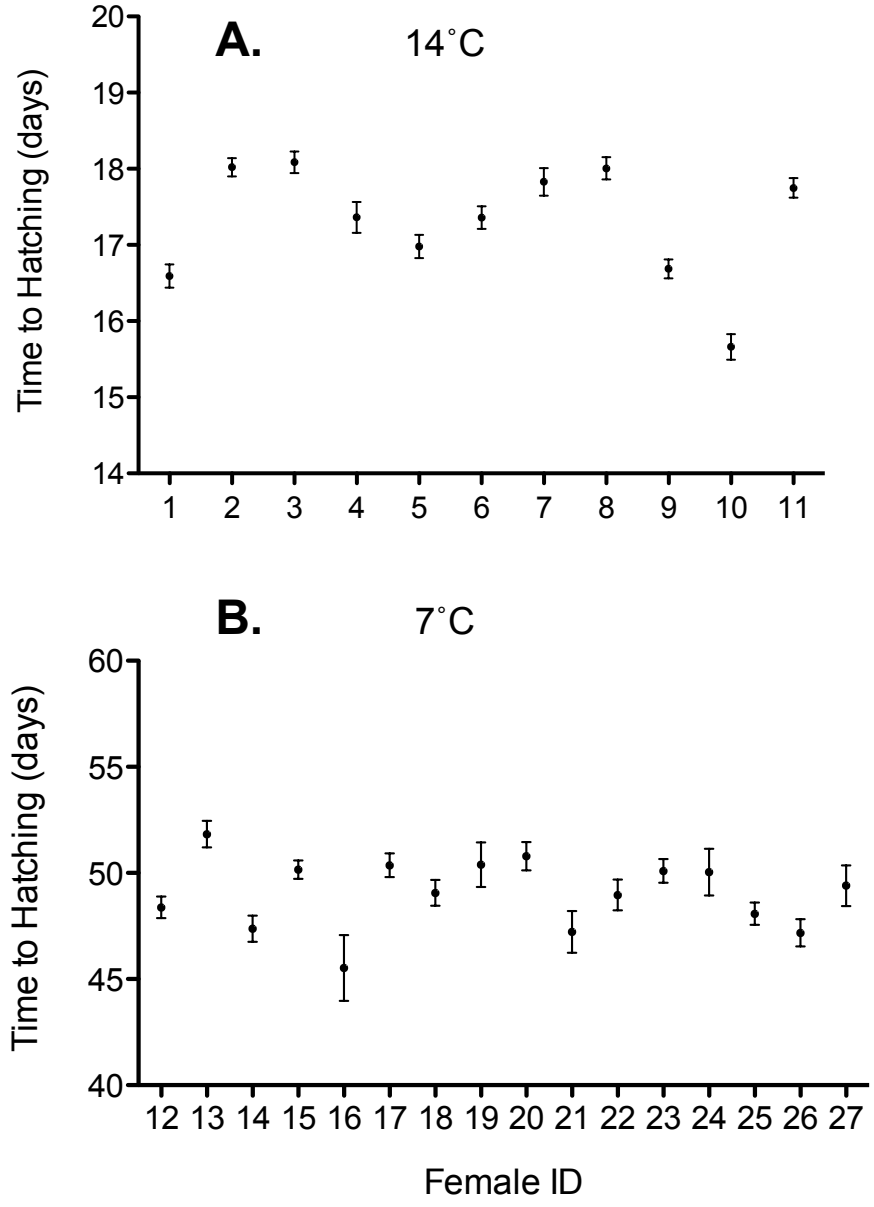

Figure 3.2. Significant variation in hatching timing among eggs of different female *Taricha granulosa* from a single population. A. Results (mean ± SE number of days) for the 11 females whose eggs were raised at 14˚C. B. Results (mean ± SE number of days) for the 16 females whose eggs were raised at 7˚C.

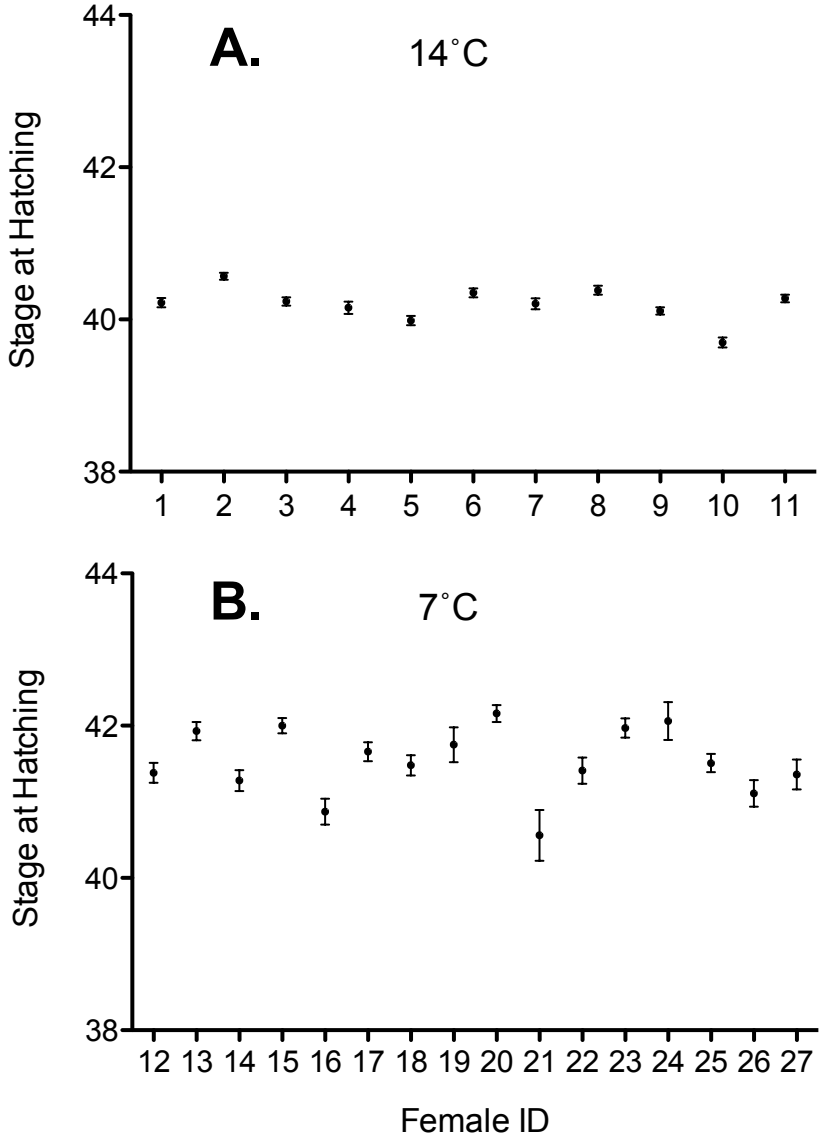

Figure 3.3. Significant variation in developmental stage at hatching among hatchlings of different female *Taricha granulosa* from a single population. A. Results (mean stage ± SE) for the 11 females whose eggs were raised at 14˚C. B. Results (mean stage ± SE) for the 16 females whose eggs were raised at 7˚C.

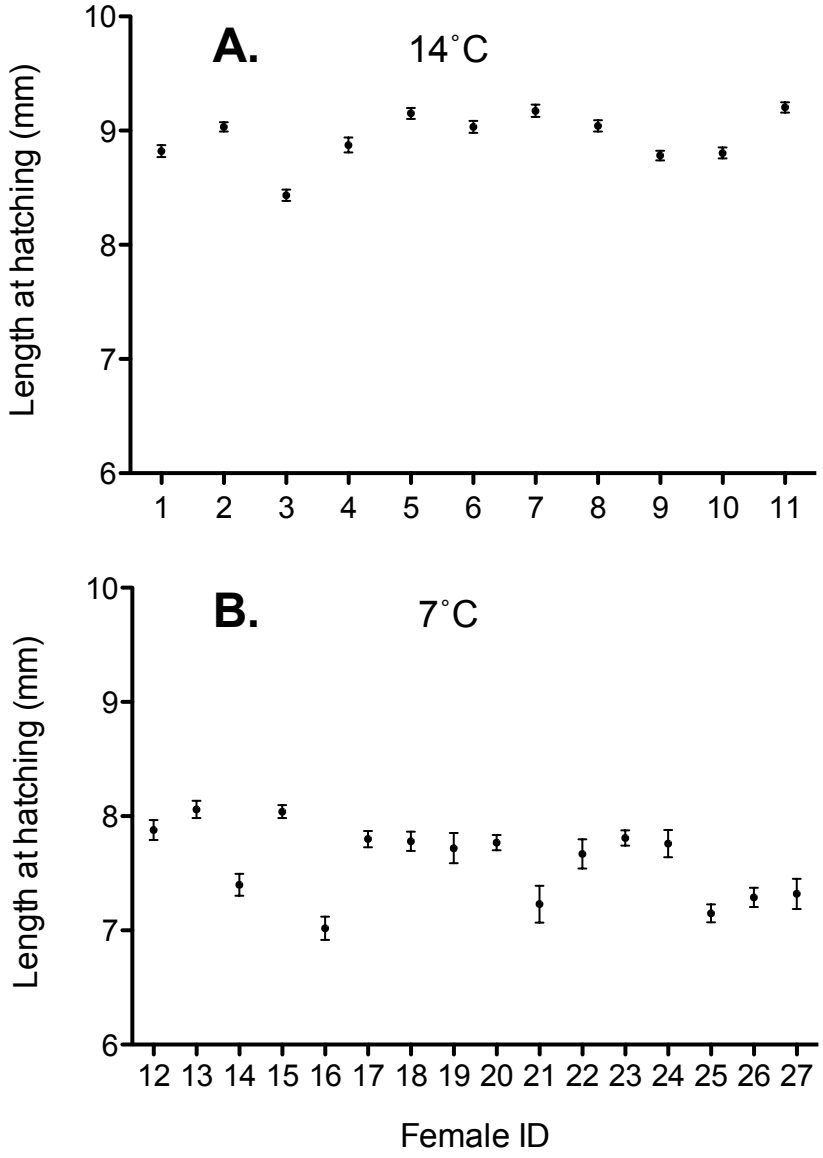

Figure 3.4. Significant variation in size (total length in mm) at hatching among hatchlings of different female *Taricha granulosa* from a single population. A. Results (mean ± SE) for the 11 females whose eggs were raised at 14˚C. B. Results (mean ± SE) for the 16 females whose eggs were raised at 7˚C.

DISCUSSION

The present study demonstrates, with 27 different females and 4746 individual eggs, significant variability in embryonic development and time to hatching among families of *T. granulosa* from a single population, and that these patterns of variation are present regardless of environmental temperature. We could not explain differences among families by female morphology or egg or clutch size.

We found a slight decrease in variability at higher temperatures, but the overall effect of family on all three response variables (i.e., hatching timing, developmental stage, size at hatching) was strong for both temperatures. Consistent with these results, other studies have also found that there is less variability in hatching timing for individuals hatching at high vs low temperatures (Voss 1993). The effect of temperature, a key environmental influence, on amphibian embryonic development, is well known (Bachmann 1969; Brown 1975; Bradford 1990; Voss 1993), and our results are consistent with previous studies. Newt embryos reared at 14˚C hatched 41.5 days sooner than those raised at 7˚C, but at an earlier developmental stage, as has been found in other amphibians (Brown 1975; Williamson & Bull 1989; Voss 1993). A similar study on the sympatric salamander *Ambystoma gracile* found a difference of 62 days in the hatching timing of eggs raised at 7 vs 12˚C (Brown 1975).

Maternal effects on the phenotype of her offspring are ubiquitous among both plants and animals, especially in relation to life-history traits (reviewed by Räsänen & Kruuk 2007). In our study, we considered two easily measurable sources of potential maternal effects that could influence early life-history traits of newts: maternal size (SVL

and weight) and mean egg diameter. We found no biologically significant effect of either trait on hatching timing, developmental stage, or size at hatching in *T. granulosa*. Female size (Travis 1983; Semlitsch & Schmiedehausen 1994; Crespi & Lessig 2004) and egg size (Kaplan 1980; Crump 1984; Kaplan 1985, 1989; Semlitsch & Gibbons 1990; Crespi & Lessig 2004; Thumm & Mahony 2005; Kaplan & Phillips 2006) significantly influence hatching timing and embryonic development in other amphibian species. However, some experiments have found mixed influences of maternal effects, with either female body size and/or egg diameter significantly correlating with some offspring life-history traits but not with others (Kaplan 1980; Travis 1983; Travis et al. 1987; Kaplan 1989; Thumm & Mahony 2005). Still, other studies found that maternal effects play little or no role in explaining significant interfamily variation in various life-history traits including hatching timing and embryonic development; in these studies genetic effects explained more of the variation (Williamson & Bull 1989; Newman 1994; Kopp & Baur 2000; Laurila et al. 2002; Alcobendas et al. 2004; Laugen et al. 2005). While many studies have found that non-genetic maternal effects explain very little variation in developmental traits, and our analyses seem to support this assertion, we cannot absolutely discount other unmeasured maternal effects, such as female lipid, hormone content or yolk quality (e.g. Crump & Kaplan 1979) from playing a role in explaining the variation we observed.

The few studies that have successfully partitioned out the genetic nature of variability in amphibians from maternal and environmental influences have found a significant underlying genetic basis for variability in larval and juvenile life-history traits (Berven 1982; Travis et al. 1987; Newman 1994; Kopp & Baur 2000; Alcobendas et al.

2004; Laugen et al. 2005). Our results, that variation among families persisted regardless of environmental temperature or measured maternal effects suggests that the variation we observed in these traits may be due to genetic differences between individual females. However, more data, including paternal identity, are needed to definitively determine if the variation we observed in hatching timing and embryonic development is genetic, and thus able to lead to evolutionary change. Regardless of its exact cause, however, the interfamily variation we observed in this study is substantial, and it is this variation that can serve as the raw material for natural selection.

For natural selection to act upon a trait, there must not only be significant variation in the trait within a population, but such variation must also confer survival advantages to some individuals but not others. The traits we examined for interfamily variation in this study are all highly adaptive. Embryonic development and hatching timing may considerably influence an individual's performance during later life stages in both invertebrates and vertebrates (Whittington & Kearn 2011; De Block et al. 2005; Warkentin 2011). In amphibians, hatching timing, size, and stage have been shown to have significant fitness consequences in both anurans (Warkentin 1995, 1999) and caudates (Boone et al. 2002) in relation to survival, the onset of feeding, and competitive and predatory interactions. The survival of *T. granulosa* larvae in particular, in predatory encounters with dragonfly nymphs, is affected by the size at which larvae hatch (Gall et al. 2011a). In addition, at the population we studied, larval caddisflies (Trichoptera) are a major predator on *T. granulosa* eggs, and have the potential to eliminate the entire reproductive output of the newt population in as little as 36 hrs (Gall et al. 2011b). The presence of interfamily variation in size, developmental stage and time

time to hatching may lead to differential survival between clutches, and therefore be critically important for the evolution of rapid development in response to selection from this predator.

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CHAPTER 4

POTENTIAL FOR LOCAL ADAPTATION IN RESPONSE TO AN ANTHROPOGENIC AGENT OF SELECTION: EFFECTS OF ROAD DEICING SALTS ON AMPHIBIAN EMBRYONIC SURVIVAL AND DEVELOPMENT[3]

The application of millions of tons of road deicing salts every winter in North America presents significant survival challenges to amphibians inhabiting road-side habitats. While much is known of the effects of NaCl on anuran tadpoles, less is known of effects on amphibian eggs, or any caudate life stage. In addition, little is known of the effects of $MgCl_2$, which is now the 2nd most commonly used road deicer. Most studies have considered amphibians to be helpless victims of deicing salts, and ignore the possibility of the evolution of local adaptation to this stressor. We attempt to address these knowledge gaps and explore this evolutionary potential by examining the effects of NaCl and $MgCl_2$ on the survival and development of eggs from different female rough-skinned newts (*Taricha granulosa*) from the same population. We demonstrate that both salts, at environmentally relevant concentrations, severely affect the embryonic survival and development of this amphibian, but that the effects of the salt are dependent on the identity of the mother. This female x treatment interaction results in substantial variation in tolerance to road deicing salts among newt families, providing the raw material necessary for natural selection and the evolution of local adaptation in this amphibian.

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INTRODUCTION

Roads are a dominant feature of the North American landscape, with approximately the same coverage area as streams (~1% of the land area of the contiguous United States; Forman 2000; Riitters and Wickham 2003). It has been estimated that 20% of the total land area of the contiguous United States is located within 127 m of a road (with only 3% located over 5 km away) (Riitters and Wickham 2003), and this proximity has been shown to have direct and indirect ecological consequences (Forman 2000; Trombulak and Frissell 2000). These include impacts on species richness (for example, with amphibians (Houlahan and Findlay 2003; Collins and Russell 2009)), mortality from construction and vehicle collision, the spread of exotic species, and the alteration of the physical and chemical environment (reviewed by Trombulak and Frissell 2000).

One of the major ways in which roads alter the chemical environment of ecosystems is through the run-off of contaminants, including heavy metals, organic pollutants, and road deicing salts. Fourteen million tons of deicing salts are estimated to be applied every winter in North America (Environment Canada 2001). This application has arguably led to the dramatic salinization of fresh water in areas of the continent over the last three decades (Kaushal et al. 2005). Chronic exposure to chloride concentrations greater than 240 mg/l has been deemed harmful (median lethal concentration) to approximately 10% of aquatic life (Environment Canada 2001; Kaushal et al. 2005), and concentrations in large excess of that limit, up to 4000 mg/l Cl^- have been found in road-side ponds and wetlands today (Environment Canada 2001). Chloride concentrations in roadside ponds have been found to greatly exceed those in ponds located away from

roads (Turtle 2000; Karraker et al. 2008; Collins and Russell 2009; Brady 2012). Traditionally, NaCl has been the primary component of road deicers. More recently, however, other deicers, such as $MgCl_2$, have been used alongside or in place of NaCl, due to their increased performance at low temperatures and decreased corrosive properties (Forman et al. 2003; Harless et al. 2011). According to a recent survey of 22 states and 3 Canadian provinces by the National Transportation Research Board (2007), liquid $MgCl_2$ is currently the 2nd most widely used chemical deicer in North America, after NaCl. Although NaCl may be the most abundantly applied salt, a study examining the leaching of road deicing salts in soils in New York State found that Mg^{2+} from $MgCl_2$ was the most abundant, reactive salt cation in roadside soils (Cunningham 2008). In addition, $MgCl_2$ is now being used exclusively by some agencies, in place of NaCl (e.g., Oregon Department of Transportation 2012; Kendal Weeks, Oregon Department of Transportation Road Maintenance, personal communication). Despite this prevalence of use, very few studies have examined the potential biological effects of this deicing salt on the inhabitants of freshwater ecosystems.

Amphibians, with their permeable skin and eggs, are an osmotically-challenged group of animals (Shoemaker and Nagy 1977). Adult tree frogs, for instance, have been recorded to lose, through evaporative passive water flux, up to 250 ml body water/kg/day, far exceeding the less than 3 ml/kg/day loss of most reptiles (Shoemaker and Nagy 1977). At the same time, most freshwater amphibians have evolved to pump ions including Na^+ and Cl^- into their bodies because they live in hypoosmotic environments (Duellman and Trueb 1994). These adaptations for acquiring and retaining ions, not for removing or restricting them, combined with the constant challenge of retaining water in the body

cavity, mean that amphibians are particularly maladapted to hyperosmotic environments, such as salt water. The survival and demography of all life-history stages of frogs and toads, specifically, have repeatedly been shown to be negatively affected by NaCl (Padhye and Ghate 1992; Viertel 1999; Dougherty and Smith 2006; Karraker et al. 2008; Collins and Russell 2009; Karraker and Ruthig 2009; Langhans 2009; Petranka and Doyle 2010; Harless et al. 2011; Alexander et al. 2012) and two recent studies suggest that $MgCl_2$ may actually be more toxic than NaCl to tadpoles (Dougherty and Smith 2006; Harless et al. 2011). Most studies on the effects of salt on amphibians have focused on the adult or larval stage, ignoring the eggs, despite some evidence suggesting that eggs may in fact be the most susceptible life-history stage to salt (Beebee 1985; Padhye and Ghate 1992; Karraker and Ruthig 2009). In addition, nearly all studies have been conducted on anuran amphibians, despite the published assertion (Neill 1958) and field and experimental evidence (Karraker et al. 2008; Collins and Russell 2009; Karraker and Ruthig 2009; Chambers 2011) that salamanders and newts (i.e., caudates) may actually be more sensitive to salt than frogs and toads. We address these gaps in our knowledge, in this paper, by examining the effects of both NaCl and $MgCl_2$ road deicing salts on the survival and development of eggs from the rough-skinned newt (*Taricha granulosa* Skilton), a common salamandrid amphibian inhabiting the west coast of North America, an area where both NaCl and $MgCl_2$ are currently used (Warrington 1998; California Department of Transportation 1999; Oregon Department of Transportation 2012; Washington State Department of Transportation 2012).

In response to environmental stressors such as road deicing salt, amphibians have traditionally been viewed as helpless players, and thus the potential for local adaptation

and the evolution of tolerance to the stressor has largely been ignored (Brady 2012). However, we are now aware that evolution can act on ecological time scales and in response to anthropogenic stressors (Carroll et al. 2007), and that local adaptation via natural selection can mediate responses to contemporary environmental change (Kawecki and Ebert 2004). Indeed, the moor frog, *Rana arvalis*, has been shown to locally adapt to acidification of its habitat (Andrén et al. 1989; Merilä et al. 2004), and recently, Brady (2012) showed that populations of the salamander *Ambystoma maculatum* appear to be locally adapted to living adjacent to roads. This local adaptation was attributed to a possible evolved tolerance of road deicing salts. The underlying basis for local adaptation is natural selection (Kawecki and Ebert 2004), and so it is first important to demonstrate that the raw material for selection, namely, variation in adaptive traits, is present in a population if one is to understand the potential for local adaptation to occur in response to a stressor. Understanding this potential, and the mechanisms behind such evolution of salt tolerance is critically important for conservation efforts (Carroll et al. 2007), especially in this era of unprecedented declines in amphibian populations worldwide (Stuart et al. 2004; Mendelson et al. 2006).

The purpose of our study was two-fold: (i) to determine the effects of two commonly used road deicing salts, NaCl and $MgCl_2$, on the embryonic survival and development of *Taricha granulosa*, and (ii) to investigate the possibility for the evolution of local adaptation in this species, by determining if significant variation in salt tolerance exists among families of newts from a single population.

MATERIALS AND METHODS

Experimental Animals

Sixteen gravid adult female rough-skinned newts (*Taricha granulosa*) were collected by hand and dip-net from the Soap Creek ponds in Benton County, Oregon (44°40'14.10"N, 123°16'37.47"W) April 4 – 5, 2011, for use in this study. These ponds are a series of eight rectangular (22.86 m x 91.44 m, 3 – 4 m deep) man-made ponds, in two rows of four, each pond separated from the next by only a 2 m grassy berm (Gall et al. 2011a). This collection of closely spaced ponds are surrounded by oak woodland and feral pasture and together represent an environmentally homogenous habitat, home to a single population of newts (Gall et al. 2011a). *Taricha granulosa* have empirically been shown to be highly philopatric to these ponds (Landreth and Ferguson 1967), and there is no appreciable genetic structuring of newt populations in this geographic area (within at least 20 km of the ponds) (Jones et al. 2001). The Soap Creek ponds are located 313 m away from the nearest paved road (a small, two lane, county road), and are separated from the road by several areas of low elevation land, which would prevent run-off from the road from reaching the ponds. Deicing salts are not applied to this road, or any of the county roads in this area (Kendal Weeks, Oregon Department of Transportation Road Maintenance, personal communication; Kent Mahler, Benton County Road Maintenance, personal communication). Salt ($MgCl_2$) is also not applied to the stretch of highway closest to the ponds (Kendal Weeks, Oregon Department of Transportation Road Maintenance, personal communication), which is located over 4 km away, at a similar

elevation, and the intervening area is covered with a series of hills not conducive to a flow of run-off from the highway reaching the ponds.

All animals were brought back to Utah State University where they were housed individually in 37.85 l glass aquaria with approximately 14 l of filtered, chilled tap water. Newts were housed in an environmental control chamber at 7˚C on a 12h : 12h light : dark cycle and fed blackworms (*Lumbriculus variegatus*) *ad libitum* prior to commencement of experiments. Methods adapted from Hopkins et al. (2012) throughout.

Females were injected with 10 µl luteinizing hormone releasing hormone ([des-Gly10, D-His(Bzl)6]-LHRH ethylamide; Sigma #L2761) to induce egg deposition and provided a small piece of polyester fiber and branch as oviposition sites. All females began depositing eggs April 10 – 15, and were finished depositing by April 22 – May 9. All eggs were collected and separated from the oviposition site within 12 hours (at which point timing of the lengths of the embryonic period began) and stored in plastic containers with chilled tap water before being assigned treatments. After all eggs were deposited, the mass and snout-vent length (SVL) of all females were recorded. Egg diameter was measured for 10 eggs per female (eggs not used in the experiment) using an ocular micrometer with an Olympus stereo-microscope.

Solution Preparation

Newt eggs were reared in seven different solutions, six treatments and one control. Control solution was composed of 20% Holtfreter's Solution, a solution recommended for the successful development of caudate embryos (Armstrong et al. 1989), which corresponds to a salinity of 0.7 g/l Cl^-. Treatment solutions were Low (1.0

g/l Cl^-), Medium (1.5 g/l Cl^-) and High (2.0 g/l Cl^-) concentrations of NaCl and $MgCl_2$, made by mixing pure biological crystalline laboratory grade salts (Thermo Fisher Scientific (New Jersey) – NaCl and Acros Organics (New Jersey) - $MgCl_2$) with distilled H_2O. These salt concentrations are well-within realistic field limits (of up to 4.0 g/l Cl^-) that have been reported in road-side ponds in North America (Environment Canada 2001), and the Cl^- concentrations (up to 2.05 g/l) reported for run-off from salted roads into aquatic habitats in parts of *Taricha granulosa*'s range (Hoffman et al. 1981). Small amounts of buffer were added to solutions if necessary to ensure the pH of all solutions was approximately neutral (7.0 – 7.5). All solutions were stored in sealed glass jars at 7˚C in the same environmental chamber as the newts until the commencement of experiments.

Experimental Procedure

Within 12 hours of oviposition each female's eggs were randomly assigned in groups of three to a lidded round plastic petri dish (hereafter "cup") (3.5 cm diameter, 1 cm deep), glued to a plastic cafeteria tray (40 x 31 cm) for stability, which was itself assigned to a random location within a 2.6 x 3.1 x 2.3 m environmental control chamber at 7˚C with a 12h light : 12h dark cycle. Each cup was randomly assigned either the control solution or one of the 6 experimental treatments. Four milliliters of this solution were pipetted into each cup and three of the assigned female newt's eggs were carefully placed in the corresponding cup. Eggs from different females were never mixed in the same cup. A line was drawn on the outside of each cup at this point to indicate the water level and cups were checked on a daily basis throughout the experiment for dehydration.

If needed, a small amount of distilled H_2O was added to cups to bring the water level up to this line again; adding distilled water only ensured that the salinity concentration of each cup remained constant throughout the experiment, as water might have evaporated, but the salt in the solution would have remained. Each cup was labeled with a unique number as well as the assigned female and treatment and date of oviposition. All eggs were deposited April 15 – May 9th, 2011. Female newts deposited between 117 – 594 eggs each (mean ± SE = 300.13 ± 36.02), corresponding to a total of 2577 eggs placed in control (859 cups), 363 eggs in Low NaCl (121 cups), 369 eggs in Low $MgCl_2$ (123 cups), 366 eggs in Medium NaCl (122 cups), 369 eggs in Medium $MgCl_2$ (123 cups), 345 eggs in High NaCl (115 cups), and 354 eggs in High $MgCl_2$ (118 cups). Assignment of eggs was weighed more heavily to control, as more animals were needed for a separate experiment on larval biology (not discussed here).

Cups were checked on a daily basis for dead eggs, which were noted and removed, keeping track of what day the egg died, who its mother was, and what treatment and cup number it was raised in. Cups were also monitored on a daily basis for egg hatching, and when hatchlings were free-swimming the date of hatching was recorded, and the hatchling removed from its cup with a pipette. The total length of each hatchling newt was then immediately measured to the nearest millimeter using an ocular micrometer attached to an Olympus stereo-microscope, and its developmental stage (Harrison 1969) recorded. Although snout-vent length (SVL) is typically measured in wild caudate populations because of the overriding issue of tail autotomy, for newly hatched newt larvae in the lab, not facing this issue, measuring the total length of

hatchlings is considered a more appropriate estimate of body size.

Statistical Analysis

To calculate the time eggs were alive, we subtracted the date eggs were deposited from the date eggs died (for eggs that died) or the date eggs hatched (for eggs that survived). For the survival data, the total number of eggs that died in each cup compared to the total number of eggs per cup was tabulated and used as the number of "events" per "trial" in a Logit-linked generalized linear model.

All analyses were conducted on egg-level data, with the random structuring effect of cup included in the model. For the normally-distributed variables of time eggs were alive (days), hatching timing (days), developmental stage at hatching, and size at hatching (mm), we used a generalized linear mixed model with two-way ANOVAs and Type III sums of squares in PROC GLIMMIX in SAS®, with the fixed effect factors of salt treatment, individual female, and their interaction. These factors were also nested within cups as a random-effect factor to account for this experimental structuring of the egg-level data. While individual female is often thought of as a random factor in analyses, we chose to treat it as a fixed-effect factor, as we were specifically interested in how the mean responses of these specific, representative females differed from each other in regards to salt tolerance. Analyzing this sort of dependency of individual female identity on the effect of salt treatment on eggs involves the creation of an interaction term that is only possible when both salt treatment and female are treated as fixed, not random effects (Bennington and Thayne 1994). We analyzed the egg survival data using logistic

regression with PROC LOGISTIC in SAS®, and Type III tests of fixed effects. We applied the Williams method (Williams 1982) to address overdispersion caused by eggs nested within cups, and a penalized maximum likelihood estimation 'Firth' correction (Firth 1993; Allison 2008) to deal with issues of quasi-separation of data caused by some cups having 100% survival or mortality. If there was an overall significant effect of treatment, we conducted Tukey-adjusted multiple comparison tests between all treatment levels to determine the effect of the different salt concentration on all variables.

If individual female was found to have a significant effect on any of the response variables, we reanalyzed our normally-distributed data to see if any visible morphological characteristics of the females could explain the interfamily variation we observed. Female mass, snout-vent length (SVL), mean egg diameter and total number of eggs laid were incorporated individually into a mixed ANCOVA model with the morphological trait in question treated as a continuous fixed effect factor and treatment as a categorical fixed effect factor. In this model only, female as a fixed effects factor is replaced by morphological trait as a continuous fixed effect factor, and female is treated as a random factor, because female identity and morphological trait of that female are completely confounded with each other when both are treated as fixed in the same model. Of specific interest in each of these trait models is the treatment by trait interaction, the significance of which could indicate that a given maternal characteristic is implicit in the significance of the female by treatment interaction in our original models.

All statistical analyses were conducted using SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA), with significance set at $\alpha = 0.05$.

RESULTS

There was a significant overall effect of salt treatment, female identity, and the interaction between treatment and female for all response variables measured (Table 4.1). There was significant underlying variation among females in all variables at the control level (Fig. 4.3 Supplemental Information), and so, for illustrative purposes, effects of salt treatments on individual females are displayed relative to effects seen at the control level (Fig.4.1BC, Fig. 4.4 Supplemental Information).

Table 4.1. The overall effect of salinity treatment, individual female, and the interaction between treatment and female on egg survival (logistic regression), time eggs alive (days), time to hatching (days), developmental stage at hatching, and size (length – mm) at hatching (two-way ANOVAs).

	Egg Survival		Time Egg Alive		Time to Hatching		Stage at Hatching		Size at Hatching	
	χ^2_{df}	*P*	F_{df}	*P*	F_{df}	*P*	F_{df}	*P*	F_{df}	*P*
Treatment	χ^2_6= 198.03	<0.0001	$F_{6,1469}$ = 61.11	<0.0001	$F_{6,1367}$ = 64.55	<0.0001	$F_{6,1366}$ = 346.69	<0.0001	$F_{6,1367}$ = 455.15	<0.0001
Female	χ^2_{15}= 139.82	<0.0001	$F_{15,1469}$ = 5.36	<0.0001	$F_{15,1367}$ = 3.79	<0.0001	$F_{15,1366}$ = 6.15	<0.0001	$F_{15,1367}$ = 9.30	<0.0001
Female x Treatment	χ^2_{90}= 115.52	0.0363	$F_{90,1469}$ = 2.48	<0.0001	$F_{89,1367}$ = 1.95	<0.0001	$F_{89,1366}$ = 2.65	<0.0001	$F_{89,1367}$ = 2.18	<0.0001

Treatment Effects

Increasing concentrations of either NaCl or $MgCl_2$ in general caused more eggs to die (Fig. 4.1A), and sooner (Fig. 4.5A Supplemental Information). Those eggs that survived the treatment generally hatched out sooner in salt treatments (Fig. 4.2A), were less well developed (Fig. 4.2B) and smaller (Fig. 4.2C). For all variables, control eggs

fared significantly better than eggs in any of the salt treatments (Figs. 4.1A,4.2). Egg survival responded to increasing salt concentration in a dose-dependent fashion, and there were no significant differences between the effects of NaCl and $MgCl_2$ (Fig. 4.1A). Eggs survived longer in NaCl vs $MgCl_2$ at high concentrations, but the effects at low concentrations were similar between the two salts (Fig. 4.5A Supplemental Information). All salt treatments caused premature hatching relative to eggs raised in control, but there did not appear to be a specific dose-dependent relationship among salt treatments, and $MgCl_2$ and NaCl caused statistically similar effects. High and medium NaCl treatments caused larvae to hatch slightly smaller and less developed than those raised in $MgCl_2$, but the effects of the two salts at low concentrations were very similar (Fig. 4.2BC).

Female Effects

There was significant, underlying variation in egg survival, time eggs were alive, time to hatching, and developmental stage and size at hatching among eggs from different females ('families') raised in control solution (Fig. 4.3 Supplemental Information), and this variation persisted under salt treatments (Fig. 4.4 Supplemental Information). The variation among families interacted with treatment, such that the magnitude of the effect of salt treatment on eggs depended on which family the animal belonged to (significant Female x Treatment interaction term, Table 4.1). The variation among families is demonstrated in Table 4.2 (Supplemental Information) by showing the minimum, maximum, and mean values for families at different treatments, with an increasing trend in interfamily variation with salt concentration.

None of the interactions of morphological female trait and salt treatment were statistically significant at $\alpha = 0.05$ (with the exception of time to hatching as a function of egg diameter), indicating that the morphological measured traits of the females probably played a relatively small part in accounting for the significant interfamily variation and female x treatment interaction we observed in our original models.

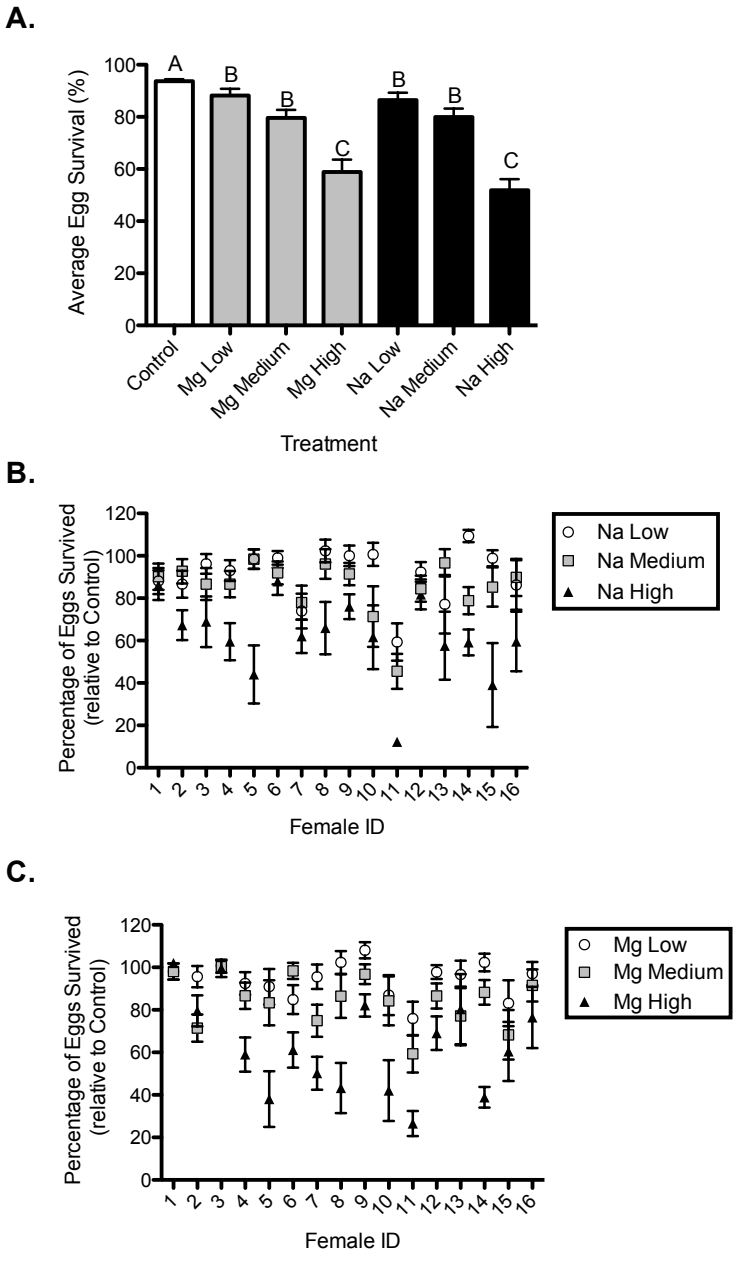

Fig. 4.1. The effect of salinity concentration (A) and maternal identity (B,C) on egg survival raised under increasing concentrations of NaCl (A,B) and $MgCl_2$ (A,C). **A.** There is a significant effect of salt treatment on mean (±SE) egg survival in each treatment (Table 4.1 for detailed statistics). Different letters indicate significant differences between treatments (Tukey-adjusted multiple comparisons). **B.** Mean (±SE) percentage of eggs survived in three increasing concentrations of NaCl relative to the survival of those eggs raised under Control conditions for 16 different female newts. There is significant variation in the response of eggs from different females to the treatments (Table 4.1 for detailed statistics). **C.** Same results for increasing concentrations of $MgCl_2$.

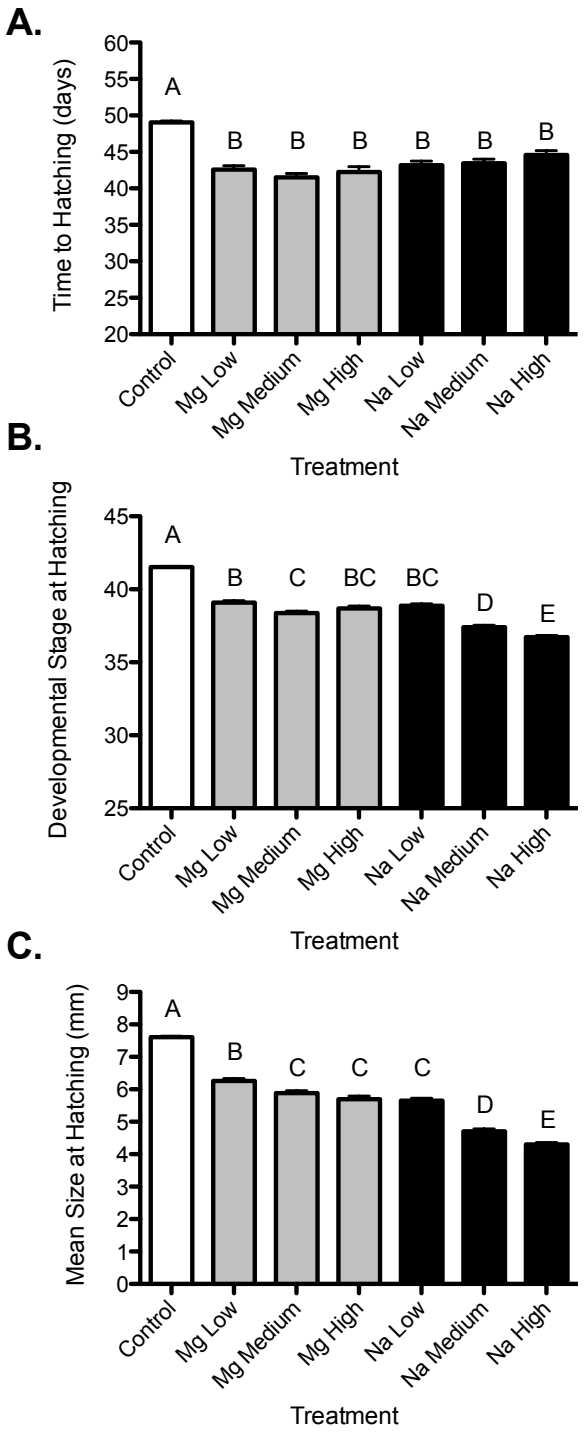

Fig. 4.2. The significant effect of salt (NaCl and $MgCl_2$) concentration on mean (±SE) **A.** Time to hatching (days), **B.** Developmental stage at hatching (Harrison 1969), and **C.** Mean size (total length – mm) at hatching. Different letters indicate significant differences between treatments (Tukey-adjusted multiple comparisons).

DISCUSSION

Road deicing salts, at environmentally relevant concentrations, caused significant mortality of newt eggs in a dose-dependent fashion. Those salt-exposed eggs that survived, hatched out sooner, and were less well developed, and at a smaller size compared to control animals. There was little difference in the overall effects of NaCl and $MgCl_2$, indicating that $MgCl_2$, an emerging stressor, is at least as toxic to *Taricha granulosa* eggs as the more traditionally used NaCl. There was significant variation among newt families within a single population in the effects of salt on all variables examined, with some families exhibiting extreme tolerance to salt (e.g., 84 – 100% survival in high salt concentrations), and others severe susceptibility (e.g., 0% survival in high salt concentrations). It appears that the raw material for natural selection to act upon is present for the evolution of local adaptation to road deicing salts in *T. granulosa*.

All life stages of amphibians have been shown to be severely affected by road deicing salts, with increased concentrations of NaCl causing egg, larval and adult mortality, and impaired growth and development (Viertel 1999; Turtle 2000; Dougherty and Smith 2006; Karraker 2007; Collins and Russell 2009; Karraker and Ruthig 2009; Langhans 2009; Petranka and Doyle 2010; Duff et al. 2011; Harless et al. 2011; Alexander et al. 2012), similar to results found for *T. granulosa* eggs in this study. The effect of $MgCl_2$ on amphibians is less well understood, despite its now prevalent use (National Transportation Research Board 2007; Cunningham 2008). We found that $MgCl_2$ was, in general, at least as toxic as NaCl. Only two recent studies (Dougherty and Smith 2006; Harless et al. 2011) have examined $MgCl_2$ toxicity on any life stage of

amphibian, and their results also indicate that $MgCl_2$ may be at least, or even more toxic to some frog tadpoles than NaCl.

Increased concentrations of both road deicing salts caused increased *T. granulosa* egg mortality, as has been found for NaCl in many other amphibian species (Beebee 1985; Padhye and Ghate 1992; Viertel 1999; Turtle 2000; Karraker et al. 2008; Karraker and Ruthig 2009; Petranka and Doyle 2010; Alexander et al. 2012). However, even those eggs that survived to hatching did not escape the negative effects of increased salt concentrations. Salt treatments caused *T. granulosa* embryos to hatch earlier, at a less developed stage and a smaller size, similar to anuran embryos exposed to NaCl (Ruibal 1959; Padhye and Ghate 1992). In some cases, we observed hatchlings that had not fully developed morphologies, presenting serious survival liabilities. However, larvae hatching with all essential structures fully developed, but earlier, or at a smaller size, may still have significant fitness consequences in relation to survival, the onset of feeding competence, and competitive and predatory interactions (Warkentin 1995,1999; Boone et al. 2002). For example, smaller, less developed *T. granulosa* larvae are more likely to be injured and die as a result of predatory attacks by dragonfly nymphs (Gall et al. 2011b).

To our knowledge, effects of road deicing salts on caudate embryonic survivorship have only been examined in one other species, the spotted salamander, *Ambystoma maculatum* (Turtle 2000; Karraker et al. 2008; Karraker and Ruthig 2009; Karraker and Gibbs 2011; Brady 2012). Similar to our results with *T. granulosa*, *A. maculatum* embryonic survival significantly declines with increasing road salt (NaCl) concentration (Karraker et al. 2008; Karraker and Ruthig 2009) and NaCl concentrations approximately analogous to our "low" salt concentrations (~1.0 g/l Cl^-) have been found

to permanently disrupt the osmoregulatory ability of *A. maculatum* eggs (Karraker and Gibbs 2011). Results with *A. maculatum* suggest that this species may be more susceptible to salt than anuran amphibians (Karraker et al. 2008; Collins and Russell 2009; Karraker and Ruthig 2009), and our results support the assertion that caudates in general may be more sensitive to salt than anurans (Neill 1958). We found severe, negative effects of salt on *T. granulosa* embryos at concentrations of 2.0 g/l Cl^- whereas studies on anuran eggs have not found negative effects until concentrations of over 4.0 g/l Cl^- are reached (e.g., Petranka and Doyle 2010; Alexander et al. 2012). Studies on *A. maculatum* by Turtle (2000) and Brady (2012) did not directly test the effects of road deicing salts, but rather compared survival in roadside versus woodland ponds, and correlated this with field measurements of increased salinity in roadside ponds. While both authors found that embryonic survival decreased significantly in roadside compared to woodland ponds, Brady (2012) also found that *A. maculatum* eggs naturally occurring in roadside ponds survived better in this environment than eggs transplanted there from woodland ponds, indicating that local adaptation to this environment may have occurred. Linking this adaptation to tolerance of road deicing salt, specifically, is however, only correlative.

The driving force behind local adaptation is natural selection (Kawecki and Ebert 2004). In order for the selection regimes necessary for the evolution of local adaptation to operate, rare alleles must exist that improve fitness in a habitat in which most individuals perform poorly (Kawecki and Ebert 2004). Our results show that environmentally relevant concentrations of salt are able to kill up to 100% of the offspring of many female newts, while the offspring from other females experience 100%

survival in this same salt concentration. For alleles promoting tolerance to exist, such variation must be under selective pressure (Kawecki and Ebert 2004). We have previously established that newt early life-history traits have the potential to be under selective pressure, in possessing the underlying interfamily variation necessary for natural selection (Hopkins et al. 2012), and we confirm and elaborate on this finding here. In the present study, we show that road deicing salt is able to act as a force of selection, by demonstrating significant interfamily variation in survival and embryonic development in response to increased concentrations of salt. We have further determined that a significant interacting effect between female genotype and saline environment exists for newt fitness, which is another prerequisite for local adaptation (Kawecki and Ebert 2004).

To what extent the variation we observed is entirely genetic, and thus able to lead to evolutionary change, remains to be determined, as we were unable to establish paternity in this study. We also cannot absolutely discount unmeasured non-genetic maternal effects such as differences in female lipid, hormone content, or yolk quality (e.g., Crump and Kaplan 1979) from contributing to the observed variation. We did try to account for some maternal effects via estimating the effects of maternal body size and weight, clutch size, and egg diameter on the observed variation. We found, similar to Brady (2012), that the measured maternal influences did not greatly influence embryonic survival or development.

Despite not knowing the extent to which the observed variation is genetic, ours is, to our knowledge, the first study to examine interfamily variation in an amphibian's tolerance to road deicing salt, which in itself is an important prerequisite for natural

selection leading to the evolution of local adaptation. Fully determining the genetic nature of this variation, as well as comparing the fitness of newt populations inhabiting road-side and non-road side ponds (Brady 2012) are obvious next steps in determining if local adaptation has indeed occurred in this amphibian species. Fully understanding and exploring this evolutionary potential is critical for conservation efforts (Carroll et al. 2007) in this time of unprecedented declines in amphibian populations worldwide (Stuart et al. 2004; Mendelson et al. 2006), and the increasing salinization of fresh water resources (Thunqvist 2004; Kaushal et al. 2005) due to an ever-expanding network of roads across the landscape (Forman 2000; Forman et al. 2003; Riitters and Wickham 2003).

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SUPPLEMENTAL INFORMATION

Table 4.2 (S1). Variation among families in (a) egg survival, (b) time to hatching (c) stage at hatching and (d) size (length) at hatching at different treatments, reporting the mean, minimum and maximum values (mean ± SE) for families for each treatment.

Treatment	Mean	Min	*N*	Max	*N*
a. Egg Survival (%)					
Control	91.50 ± 1.86	73.98 ± 2.73	321	97.96 ± 1.78	78
Na Low	82.87 ± 3.53	50.00 ± 11.34	25	96.01 ± 5.77	14
Mg Low	85.78 ± 2.15	66.56 ± 10.43	25	97.23 ± 3.98	21
Na Medium	76.76 ± 3.90	36.15 ± 10.75	25	95.34 ± 6.76	12
Mg Medium	75.99 ± 3.57	50.00 ± 11.34	24	96.79 ± 4.61	18
Na High	53.31 ± 4.92	2.88 ± 4.13	20	83.18 ± 8.12	26
Mg High	54.54 ± 6.08	17.17 ± 8.44	25	97.56 ± 3.50	24
b. Time Eggs Alive (days)					
Control	47.72 ± 0.46	44.67 ± 0.75	321	49.87 ± 1.73	58
Na Low	42.29 ± 0.56	38.00 ± 2.71	24	46.02 ± 2.23	35
Mg Low	41.12 ± 0.82	35.82 ± 2.58	26	48.78 ± 4.43	9
Na Medium	43.22 ± 1.17	35.13 ± 2.23	35	51.58 ± 3.83	12
Mg Medium	39.43 ± 0.84	34.67 ± 2.71	24	45.50 ± 3.83	12
Na High	46.89 ± 1.55	34.24 ± 2.19	38	59.50 ± 3.83	12
Mg High	33.31 ± 1.47	22.36 ± 2.31	33	42.49 ± 3.49	14
b. Time to Hatching (days)					
Control	49.04 ± 0.41	45.58 ± 1.01	79	51.72 ± 0.61	232
Na Low	43.16 ± 0.63	39.69 ± 1.78	23	47.74 ± 1.46	32
Mg Low	42.58 ± 0.71	37.56 ± 3.01	9	48.78 ± 3.01	9
Na Medium	43.44 ± 0.80	39.80 ± 2.64	11	51.58 ± 2.60	12
Mg Medium	41.50 ± 0.57	38.36 ± 1.62	28	47.66 ± 2.67	10
Na High	41.47 ± 2.96	35.50 ± 5.49	2	52.45 ± 1.70	21
Mg High	42.25 ± 0.97	38.59 ± 4.00	4	52.99 ± 4.13	3
c. Stage at Hatching					
Control	41.53 ± 0.11	40.57 ± 0.27	60	42.14 ± 0.13	238
Na Low	38.88 ± 0.14	37.47 ± 0.56	14	39.78 ± 0.71	9
Mg Low	39.10 ± 0.22	37.5 ± 0.71	9	41.11 ± 0.71	9
Na Medium	37.40 ± 0.29	34.86 ± 0.45	21	38.89 ± 0.63	11
Mg Medium	38.37 ± 0.22	36.46 ± 0.63	10	40.08 ± 0.66	9
Na High	34.09 ± 2.29	34.49 ± 0.81	5	37.98 ± 0.69	7
Mg High	38.69 ± 0.20	37.33 ± 0.41	22	40.14 ± 0.99	3
d. Size at Hatching (mm)					
Control	7.61 ± 0.08	7.03 ± 0.14	79	8.05 ± 0.08	191
Na Low	5.65 ± 0.11	4.35 ± 0.21	30	6.28 ± 0.20	32
Mg Low	6.26 ± 0.10	5.53 ± 0.26	21	7.08 ± 0.41	9
Na Medium	4.70 ± 0.15	3.37 ± 0.26	21	5.44 ± 0.23	24
Mg Medium	5.88 ± 0.12	5.16 ± 0.28	15	6.61 ± 0.38	9
Na High	3.95 ± 0.31	3.18 ± 0.47	5	5.03 ± 0.27	15
Mg High	5.69 ± 0.12	4.88 ± 0.41	6	6.51 ± 0.43	7

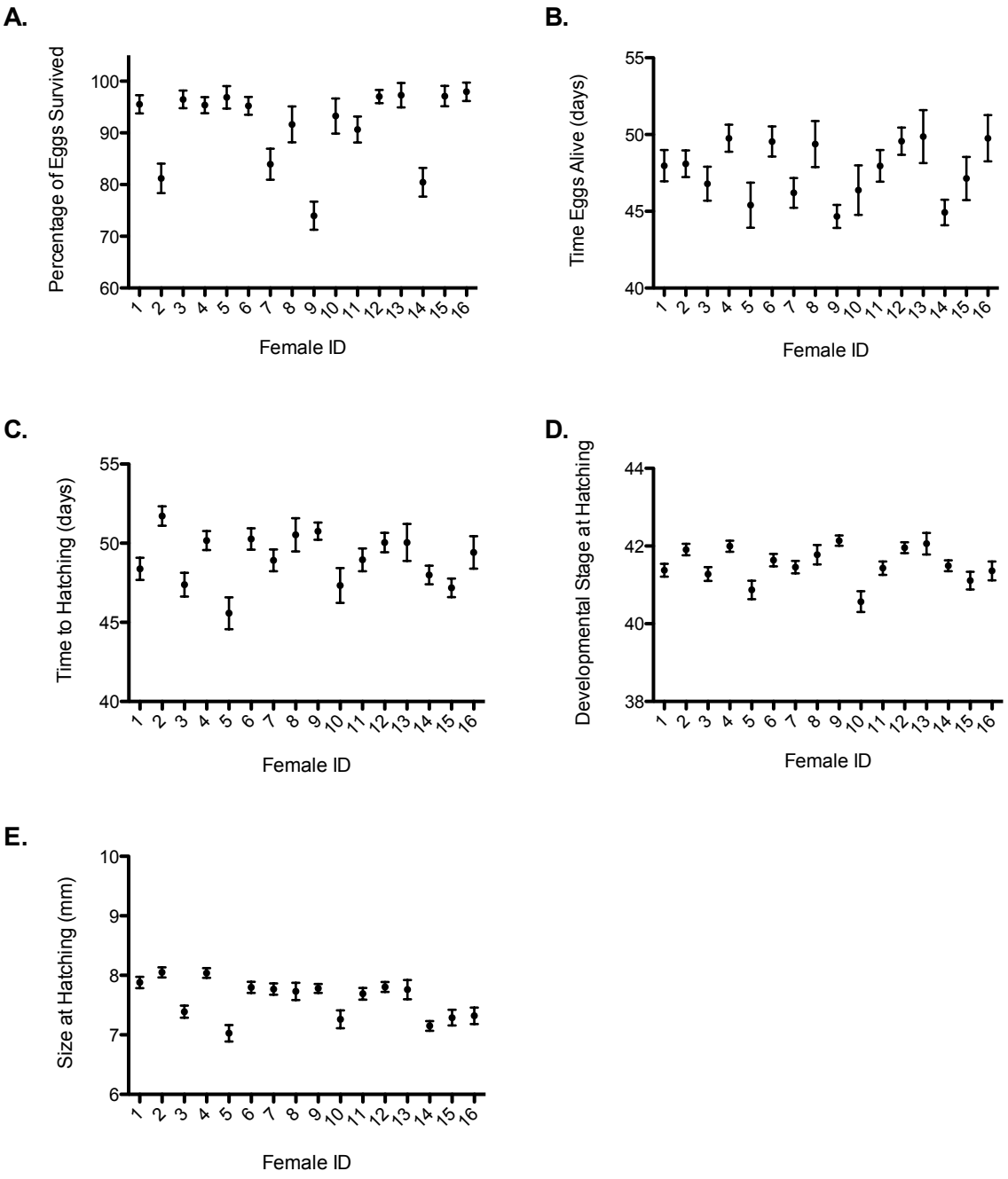

Fig. 4.3 (S1)**.** The interfamily variation in **A.** Percentage of eggs survived, **B.** Time eggs survived (days), **C.** Time to hatching (days), **D.** Developmental stage at hatching, and **E.** Size (length) at hatching (mm) for the offspring of 16 different female newts raised in Control solution. Data are mean (±SE) values for all of a specific female's offspring raised in that Control treatment. There is significant variation among females for all metrics (A-D) examined. Figures C-E reprinted from Hopkins *et al*. (2012) (Chapter 3).

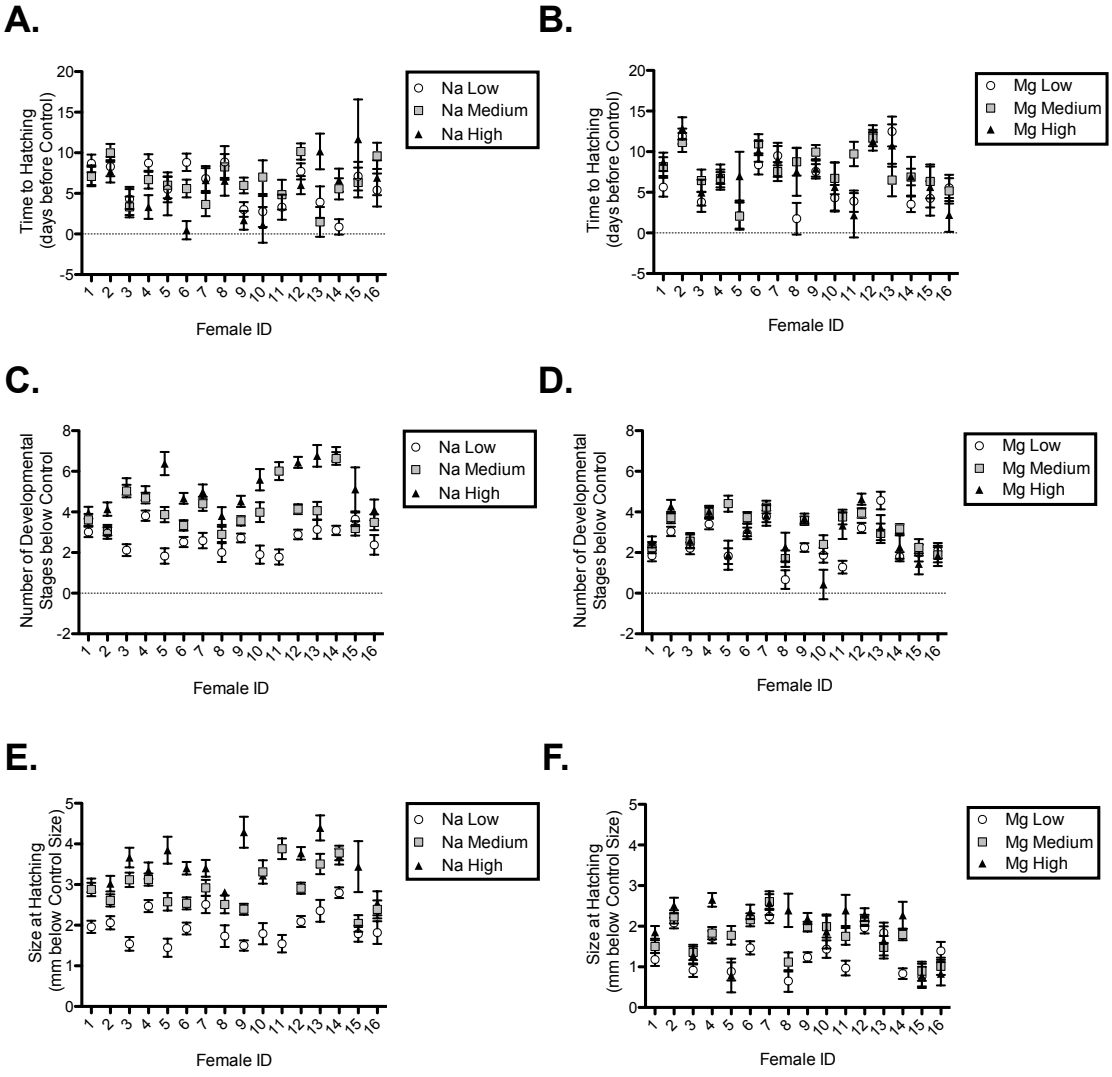

Fig. 4.4 (S2)**.** Significant variation in the response of eggs from different females to NaCl (A,C,E) and $MgCl_2$ (B,D,F) salt concentrations for **A,B.** Time to hatching (days before eggs hatching in Control), **C.D.** Developmental stage at hatching (number of developmental stages below larvae hatching in Control), and **E.F.** Size (total length in mm) at hatching (mm below the size of larvae hatching in control).

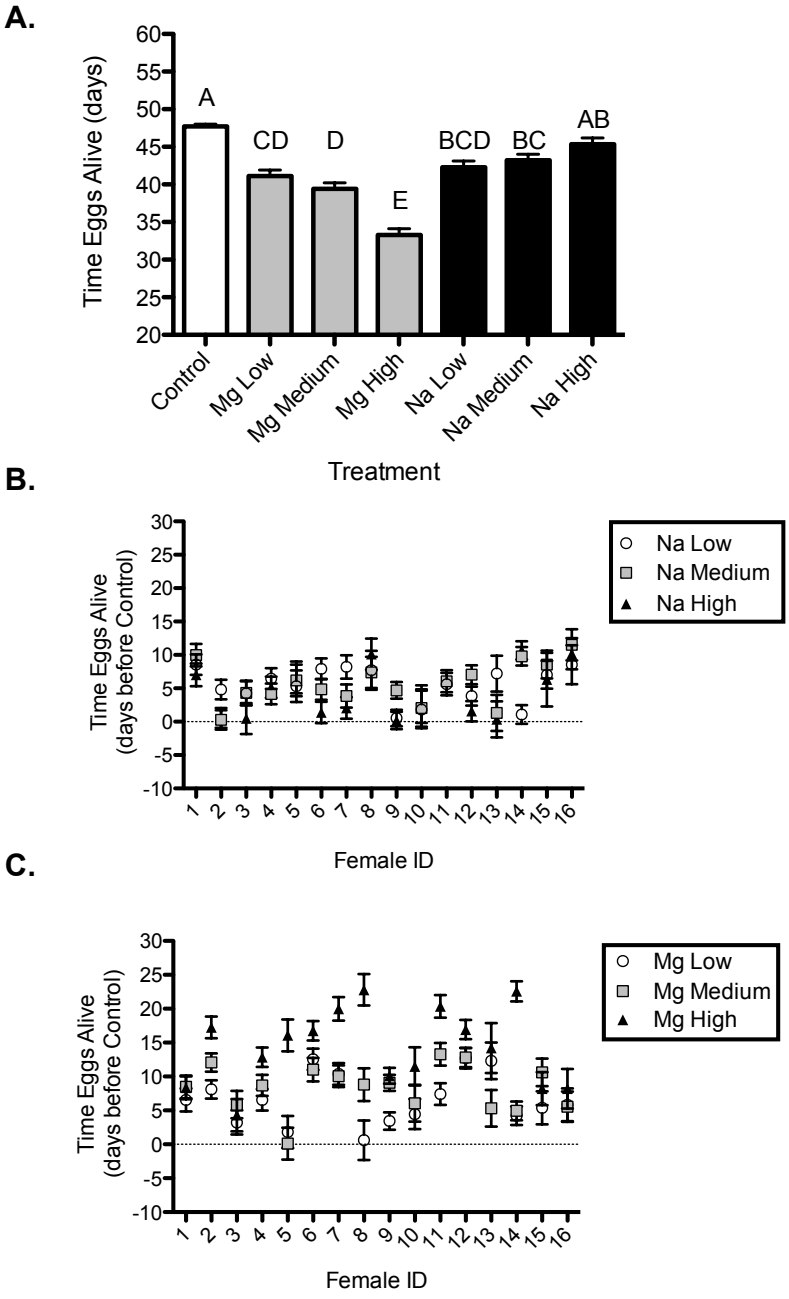

Fig. 4.5 (S3). The effect of salinity concentration (A) and maternal identity (B,C) on time eggs survived (days) raised under increasing concentrations of NaCl (A,B) and $MgCl_2$ (A,C). **A.** There is a significant effect of salt treatment on the mean (±SE) number of days eggs survived. Different letters indicate significant differences between treatments (Tukey-adjusted multiple comparisons). **B.** Mean (±SE) number of days (before those eggs in Control solution) eggs survived in three increasing concentrations of NaCl for 16 different female newts. There is significant variation in the response of eggs from different females to the treatments (Table 1 for detailed statistics). **C.** Same results for three increasing concentrations of $MgCl_2$.

CHAPTER 5

INCREASED FREQUENCY AND SEVERITY OF DEVELOPMENTAL DEFORMITIES IN ROUGH-SKINNED NEWT (*TARICHA GRANULOSA*) EMBRYOS EXPOSED TO ROAD DEICING SALTS (NaCl & $MgCl_2$)[4]

Road-side aquatic ecosystems in North America are annually polluted with millions of tons of road deicing salts, which threaten the survival of amphibians which live and breed in these habitats. While much is known of the effects of NaCl, little is known of the second most-commonly used deicer, $MgCl_2$, which is now used exclusively in parts of the continent. Here we report that environmentally relevant concentrations of both NaCl and $MgCl_2$ cause increased incidence of developmental deformities in rough-skinned newt hatchlings that developed embryonically in these salts. In addition, we provide some of the first quantification of severity of different deformities, and reveal that increased salt concentrations generally increase both deformity frequency and severity. Our work contributes to the growing body of literature that suggests salamanders and newts are particularly vulnerable to salt, and that the emerging pollutant, $MgCl_2$ is comparable in its effects to the more traditionally-used NaCl.

Capsule: Two commonly used road deicing salts, NaCl and $MgCl_2$, caused increased frequency and severity of developmental deformities in rough-skinned newt embryos.

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INTRODUCTION

Approximately 20% of the total land area of the contiguous United States is located within 127 m of a road, and only 3% is located over 5 km away (Riitters and Wickham, 2003). This coverage area of roads has profound ecological consequences (Forman, 2000; Trombulak and Frissell, 2000). For example, amphibian species richness is negatively correlated with road proximity (Collins and Russell, 2009; Houlahan and Findlay, 2003), while simultaneously, probability of skeletal deformities in these animals is positively correlated with road proximity (Reeves et al., 2008). One of the primary ways in which roads affect ecosystems is through the alteration of the chemical environment due to chemical run-off (Forman et al., 2003; Trombulak and Frissell, 2000).

Approximately 14 million tons of road deicing salts are applied annually to roads in North America (Environment Canada, 2001), principally as NaCl and $MgCl_2$ (National Transportation Research Board, 2007; Forman et al., 2003), and salt run-off from these roads has arguably led to the salinization of fresh-water resources in parts of the continent over the last several decades (Kaushal et al., 2005). Roadside habitats have consistently higher Cl^- concentrations than non-roadside habitats (Brady, 2012; Collins and Russell, 2009; Karraker et al., 2008; Turtle, 2000), sometimes reaching limits of up to 4000 mg/L Cl^-, far in excess of the recommended maximum 220 mg/L considered harmful to 10% of aquatic life (Environment Canada, 2001).

A variety of vertebrate and invertebrate organisms inhabiting roadside aquatic habitats are adversely affected by deicing salt contamination (Benbow, 2004; Blasius and

Merritt, 2002; Collins and Russell, 2009; Petranka and Doyle, 2010). Amphibians, with their permeable skin and eggs are a particularly vulnerable group of animals that live and breed in these habitats. Sodium chloride in particular has been shown to negatively affect survival of all life stages of frogs and toads (Alexander et al., 2012; Collins and Russell, 2009; Dougherty and Smith, 2006; Harless et al., 2011; Karraker and Ruthig, 2009; Langhans et al., 2009; Padhye and Ghate, 1992; Petranka and Doyle, 2010; Sanzo and Hecnar, 2006; Viertel, 1999). Much less is known of the effects of salt on salamanders and newts. To our knowledge, only one species, the spotted salamander (*Ambystoma maculatum*), has been studied (Brady, 2012; Karraker and Gibbs, 2011; Karraker et al., 2008; Karraker and Ruthig, 2009; Turtle, 2000). Results from these studies suggest that caudates may actually be more susceptible to salt than anurans, but data on other caudate species are needed in order to determine if this pattern holds true.

Compared to work on larval amphibians, relatively few studies have examined the possible effects of road deicing salts on amphibian eggs, despite some evidence suggesting that embryos may be more sensitive to salt than larvae (Beebee, 1985; Karraker and Ruthig, 2009; Padhye and Ghate, 1992). In addition to salt killing amphibian eggs, it can also cause embryos to hatch earlier, smaller, and less developed (Gosner and Black, 1957; Hopkins et al., 2012a; Padhye and Ghate, 1992; Ruibal, 1959), which in itself can have important fitness consequences (Boone et al., 2002; Warkentin, 1995, 1999). Increased developmental deformities, including cysts and spinal and gill malformations, have also been linked to embryonic development in saline water (Gosner and Black, 1957; Haramura, 2007; Harless et al., 2011; Karraker and Ruthig, 2009; Padhye and Ghate, 1992; Ruibal, 1959). Most of these studies, however, only mention

deformities anecdotally, and have not quantified frequency or severity. In addition, with the exception of Karraker and Ruthig (2009), who examined *A. maculatum* hatchlings, all studies have focused exclusively on anuran amphibians. Finally, all studies on salt-induced developmental deformities have so far dealt only with NaCl, while the second most commonly used road deicer, $MgCl_2$ (National Transportation Research Board, 2007), has been ignored. While little is known of the effects of $MgCl_2$ on amphibians in general, there is some evidence that it may be at least as, if not more, harmful to anuran tadpoles than NaCl (Dougherty and Smith, 2006; Harless et al., 2011). It is unknown if $MgCl_2$ can cause developmental deformities in amphibian embryos, or how these deformities compare to those seen from NaCl. Given the widespread use of $MgCl_2$ on the landscape (National Transportation Research Board, 2007; Cunningham et al., 2008), and the fact that some agencies are now using it exclusively (e.g., Oregon Department of Transportation, 2012), this represents a significant gap in our knowledge of effects of this contaminant.

The objective of this study was to determine if exposing eggs of the rough-skinned newt (*Taricha granulosa*), a common salamandrid amphibian inhabiting the west-coast of North America, to environmentally relevant concentrations of NaCl and $MgCl_2$ road deicing salts caused developmental deformities in hatchling newts, and to quantify the frequency and severity of these deformities as they relate to salt concentration.

MATERIALS & METHODS

Methods used to rear eggs are based on those presented in detail in Hopkins et al. (2012b). This paper should be consulted for detailed methodological explanations regarding rearing newt eggs in petri dishes in the laboratory. Methods regarding the effects of road deicing salts are based on Hopkins et al. (2012a), which should also be consulted for more detailed methodologies. Briefly, 16 gravid female newts were collected from the Soap Creek ponds in Benton County, Oregon, in the spring of 2011, and transported back to the lab at Utah State University, where they were induced to lay eggs. These ponds represent a homogenous environment which is not subjected to road deicing salts, as they are located 313 m away from the nearest paved road, which, like all other county roads in this area, is not salted (Kendal Weeks, Oregon Department of Transportation Road Maintenance, personal communication; Kent Mahler, Benton County Road Maintenance, personal communication). In addition, salts are not applied to the nearest highway (over 4 km away) (Kendal Weeks, Oregon Department of Transportation Road Maintenance, personal communication), which is at a similar elevation, and separated from the ponds with a series of hills not conducive to road run-off flow. For more information about this habitat see Gall et al. (2011) and Hopkins et al. (2012a).

Solution Preparation and Experimental Procedure

Eggs from female newts were randomized to 7 different treatments: Low (1.0 g/L Cl^-), Medium (1.5 g/L Cl^-) and High (2.0 g/L Cl^-) concentrations of NaCl and $MgCl_2$ salts

and a control (20% Holtfreter's solution; 0.7 g/L Cl^- ; a solution recommended for the successful development of salamander embryos (Armstrong et al. 1989)). These salt concentrations were chosen as they are well-within Cl^- concentrations (up to 4.0 g/l Cl^-) measured in road-side ponds impacted by deicing salts in North America (Environment Canada, 2001). Salt treatments were made by mixing pure biological crystalline laboratory grade salts (Thermo Fisher Scientific (New Jersey) – NaCl and Acros Organics (New Jersey) – $MgCl_2$) with distilled water. HCl or NaOH buffer was added in small amounts if necessary to ensure the pH of all solutions was approximately neutral (7.0-7.5). Four milliliters of each salt solution was pipetted into a 3.5 cm diameter, 1 cm deep, round, plastic petri dish, into which 3 randomly assigned eggs from a randomly assigned female were placed. These eggs were then raised at 7˚C in a randomized spot in an environmental control chamber with a 12 h light : 12 h dark cycle until hatching. Eggs were checked every day for mortality and hatching, and dead eggs and hatchlings were removed. In addition, we ensured the water level and salinity of the solutions in the petri dishes stayed constant throughout the experiment by drawing a line on the outside of the petri dish to indicate the water level, and refilling it with distilled water when necessary to replace water that was lost through evaporation. Adding distilled water ensured that the salinity remained constant, as distilled water has a salinity of 0.0 g/L (G.R.H. unpublished data), and although water may have evaporated from the dishes, the salt would have remained. Water levels were checked and corrected (if necessary) on a daily basis for the duration of the experiment.

Examination and Quantification of Deformities

Immediately upon hatching, we transferred each larva to a petri dish with distilled water, and examined it for developmental deformities under an Olympus stereo microscope. We noted the presence of any deformity and characterized its nature. We constructed a list of all deformities observed (Table 5.1), and characterized each in reference to this list. Each deformity was then scored on an increasing scale of severity from 0-3 (0 being the absence of deformity, 3 being a deformity severely detrimental to survival), with animals experiencing multiple deformities being classified according to the most severe deformity present (Table 5.1). Deformity severity was scored in such a manner based on the deformity's perceived relevance to larval survival.

Table 5.1. List of larval deformities and their associated severity score. (1 = least severe, 3= most severe). For animals that experienced multiple deformities, the highest score is used.

Deformity	Score
Slightly bent	1
Small cyst	1
Reduced limb-bud on one side	1
Split tail	1
Bent tail	1
Bent body	2
Cyst	2
Reduced gills	2
Reduced limb-buds	2
Reduced gills	2
Missing limb-buds	2
Very bent tail	2
Very bent body	3
Multiple cysts	3
Large cyst	3
Missing gills	3
Shrunken head	3
Missing eyes	3
Torn/broken yolk sac	3
Missing tail / back end of abdomen	3
Bleeding / bruised	3
Two heads	3
Acephalous	3

Statistical Analysis

For our first analysis (presence of deformities), eggs within petri dishes were treated as subsamples, and the petri dish was the replicating factor. The 16 female newts laid 117 – 594 eggs each (mean ± SE = 300.13 ± 36.02), for a total of 4701 eggs, and these were equally distributed among the 6 different salt treatments. More eggs were placed in control versus the other treatments, as larvae were needed for a separate experiment on this life-history stage (not discussed here). This distribution of eggs resulted in a total of 859 dishes (3 eggs each) of control, 121 of Low NaCl, 123 Low $MgCl_2$, 122 Medium NaCl, 123 Medium $MgCl_2$, 115 High NaCl, and 118 High $MgCl_2$. A total of 881 eggs died in this experiment (268 in control, 64 in Low NaCl, 49 in Low $MgCl_2$, 89 in Medium NaCl, 90 in Medium $MgCl_2$, 155 in High NaCl, and 166 in High $MgCl_2$). An analysis of the effect of salt treatment on egg survival is provided in Hopkins et al. (2012a). For the purposes of the present paper, we analyzed the effect of salt treatment on presence and severity of deformities found on those eggs (3819) that survived to hatching. Eggs from different females were kept separately, and the effect of individual female was treated as a random factor in the analysis. The effect of salt treatment was analyzed as a fixed-effect factor. We used these factors to predict the presence of developmental deformities using a generalized linear model with a binomial distribution and a logit link function with Proc GLIMMIX in SAS®. Where overall significant effects of salt treatment were found, we conducted Tukey-adjusted pairwise comparisons between treatment levels to compare their influences on deformity presence and severity.

We analyzed the effect of salt treatment on deformity severity score (4 levels: 0, 1, 2, 3) using an ordinal multinomial mixed model with a cumulative logit link; data analysis was generated using the GLIMMIX procedure in SAS®. Salt treatment was incorporated as a fixed effect; female and female by treatment interaction were random effects. It was not feasible to accommodate the clustering of eggs within cups in the model design structure because the high mortality of eggs in salt treatments resulted in a pronounced sparseness of hatchling deformity severity data (see Fig 5.1), hence hatchling counts were combined over all cups within each female-treatment combination. Where an overall significant effect of salt treatment was found, we estimated odds ratios for all pairwise combinations of treatment levels (salt concentrations), using a Shaffer-simulated TCTP adjusted *P*-value to judge statistical significance (Westfall and Tobias 2007; Royen 1989). Each odds ratio was estimated as the odds of a higher deformity severity score (rather than a lower deformity severity score) for one treatment level relative to the odds of a higher deformity severity score for the other treatment level. An odds ratio of one indicates no difference between treatments.

All statistical analyses were conducted using SAS® software version 9.3 (SAS Institute Inc., Cary, NC, USA), with significance set at $\alpha = 0.05$.

RESULTS

A total of 881 eggs died in this experiment, with increasing road deicing salt concentration causing increasing egg mortality (Fig 5.1; see Hopkins et al. 2012a

(Chapter 4) for complete analysis of egg mortality). For those eggs that survived to hatching, there was a significant effect of road deicing salt treatment ($F_{6,1455} = 137.31$, $P < 0.0001$) on the presence of developmental deformities in hatchlings (Fig 5.1). Only 6.12 ± 0.67 % of newt embryos that successfully hatched in control had developmental deformities, which was significantly less than newt hatchlings in any of the salt treatments (Tukey-adjusted pair-wise comparisons) (Fig 5.1). In contrast, 33.58 ± 3.27 % and 28.98 ± 3.03 % of embryos that survived in low NaCl and low $MgCl_2$, respectively, hatched with developmental deformities (Fig 5.1). There was no significant difference between these two salts (Tukey-adjusted pair-wise comparisons), but both were significantly lower in their deformity frequency than eggs that survived in medium salts (NaCl = 65.18 ± 3.40 %, $MgCl_2$ = 46.53 ± 3.59 %). The majority (73.89 ± 3.59 % in NaCl, 60.47 ± 4.08 % in $MgCl_2$) of eggs that survived in high road deicing salt concentrations had developmental deformities, with no significant differences in effects between salt types (Tukey-adjusted multiple comparisons) (Fig 5.1). Only at medium concentrations were the effects of NaCl and $MgCl_2$ significantly different from each other.

A wide variety of developmental deformities was observed, varying in their severity and frequency (Fig 5.2; Table 5.1). Most deformities were abdominal or spinal, but we also observed many cases of the reduction or absence of growth of certain tissues (e.g. gills, eyes, limb-buds, and even the head; Table 5.1). Figure 5.2 shows some photographic examples of deformity types.

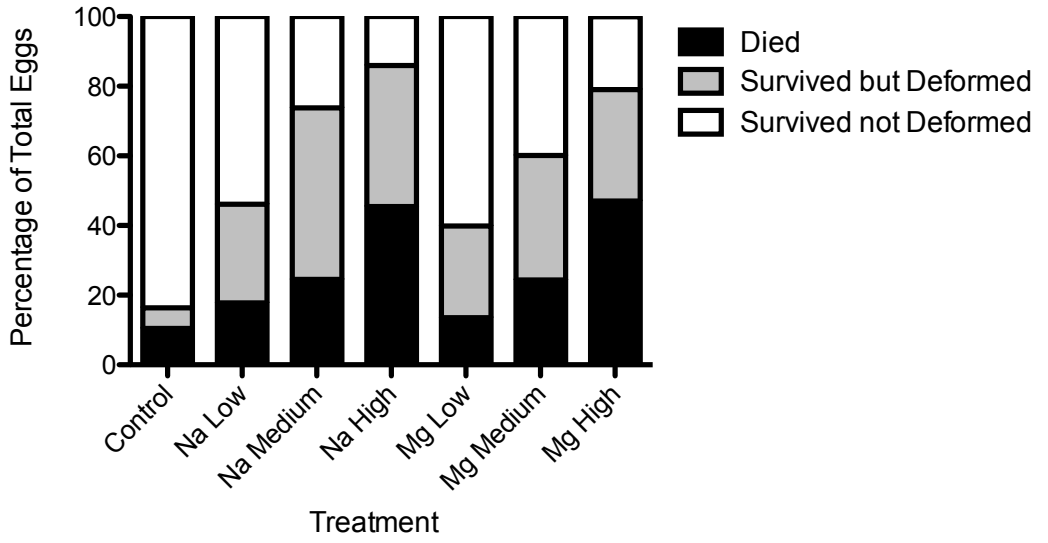

Fig. 5.1. Fate of *Taricha granulosa* eggs raised in three increasing concentrations of NaCl and $MgCl_2$ road deicing salts, and a control. Black bars indicate the percentage of eggs that died, while gray/white bars indicate eggs that survived to hatching. For those eggs that survived to hatching, increasing salt concentrations caused an increase in the percentage of embryos that hatched with developmental deformities (gray bars), vs developed normally (white bars) (see Results for relevant statistics).

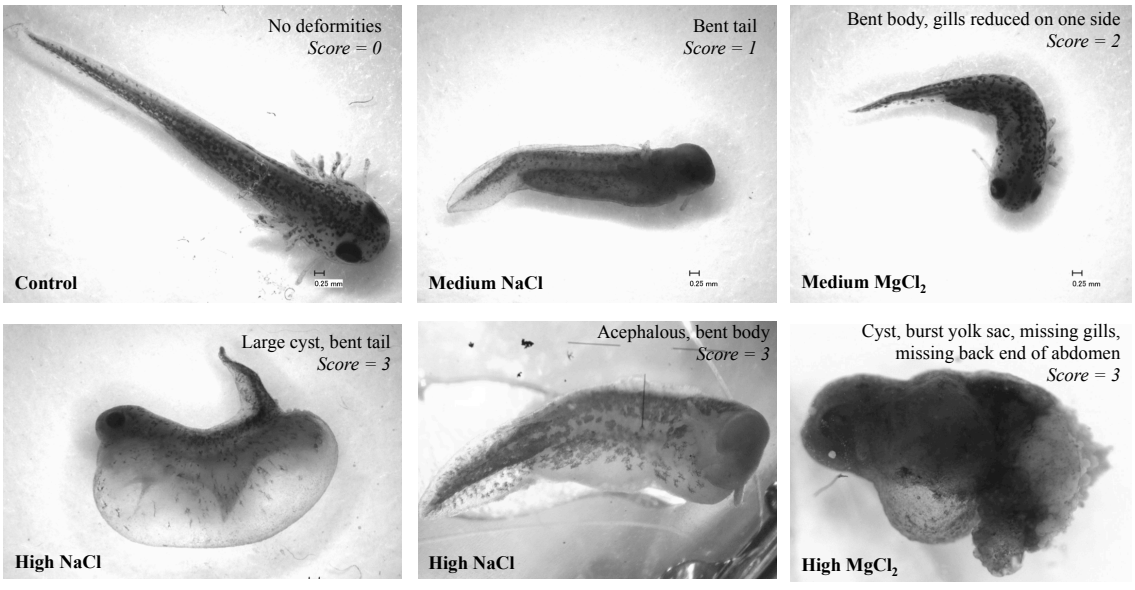

Fig. 5.2. Photographic examples of developmental deformities in each score class.

There was a significant effect of salt treatment on deformity severity score ($F_{6,89}$ = 66.97, P<0.0001), with increasing concentrations of both salts generally causing more severe deformities (Fig. 5.3, Table 5.2). For example, eggs raised in high NaCl were over 49 times more likely to develop a more severe deformity than eggs raised in control, whereas eggs raised in low NaCl were only 8.79 times more likely to develop more severe deformities than those raised in control (Table 5.2). There was little difference in the effects on deformity score between the two types of deicing salts, with only medium NaCl having a statistically greater effect on deformity score than medium $MgCl_2$ (Table 5.2). Nearly 37% of eggs that survived to hatching in high NaCl, and nearly 25% of those hatching in high $MgCl_2$, developed deformities of score 3, whereas less than 2% of eggs hatching in control developed deformities this severe (Fig 5.3).

Table 5.2. Odds ratios for pairs of treatment levels based on the cumulative logit model, and tests of whether odds ratios are different than one with *P*-values adjusted for multiplicity using the TCTP method. Each odds ratio is estimated as the odds of a higher deformity score (rather than a lower deformity score) for the horizontal treatment listed in each pair relative to the odds of a higher deformity score for the vertically listed treatment. For example, the odds of a higher deformity score for Na Low are 8.79 times greater than the odds of a higher deformity score for Control. An odds ratio of one indicates no difference between treatments.

	Na Low	Na Medium	Na High	Mg Low	Mg Medium	Mg High
Control	8.79 ***	32.61 ***	49.15 ***	7.34 ***	15.36 ***	28.05 ***
Na Low		3.71 ***	5.59 ***	1.20 n.s.	0.57 n.s.	0.31 ***
Na Medium			1.51 n.s.	4.45 ***	2.12 **	1.16 n.s.
Na High				6.70 ***	3.20 ***	1.75 n.s.
Mg Low					2.10 *	3.83 ***
Mg Medium						1.83 *

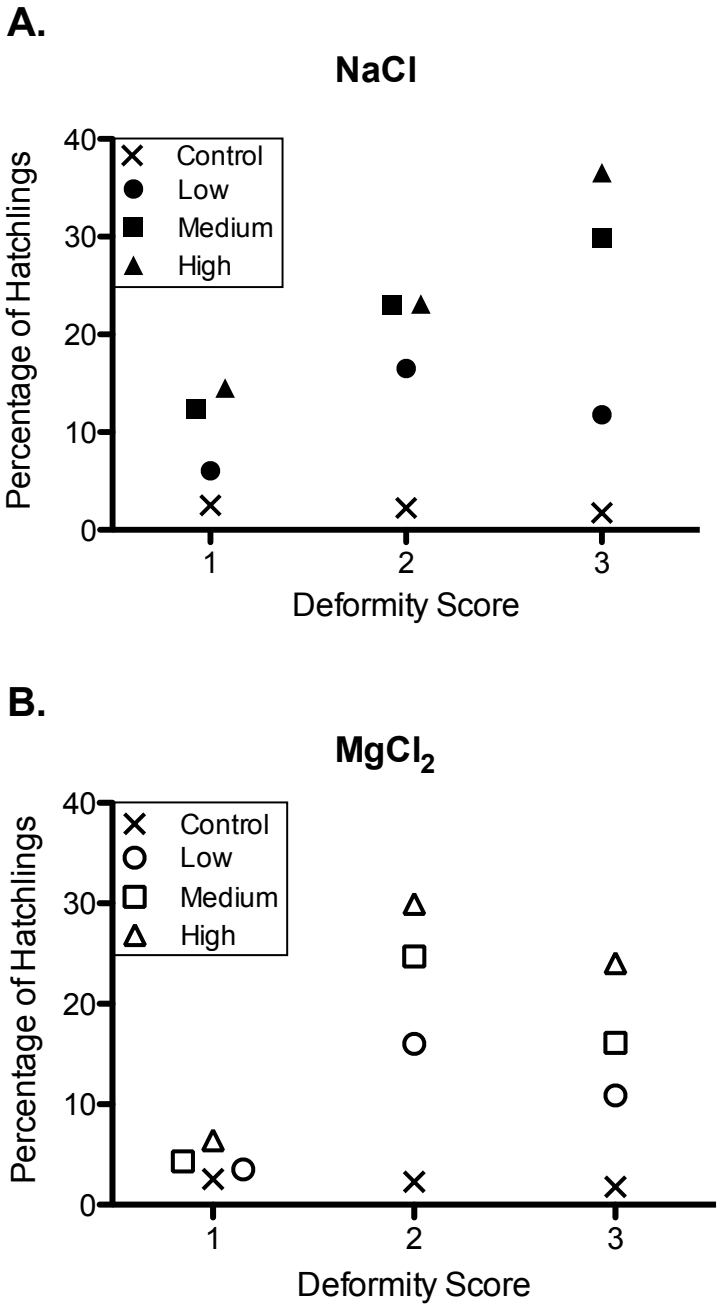

Fig. 5.3. Percentage of *T. granulosa* hatchlings (those eggs that survived) in each deformity severity score (higher scores indicate more severe deformities), raised in (A.) NaCl, and (B.) $MgCl_2$.

DISCUSSION

Salt concentrations caused a variety of deformities in newt hatchlings, including spinal deformities (bent tails and bodies), cysts, and shrunken or missing limbs and organs (gills, limb-buds, heads, eyes). In addition, many of these deformities were present in combination with others, presumably adding additional stress to the animal. Other studies examining the effects of salt on amphibian development have noted similar deformities occurring with NaCl. Spinal deformities including kinked tails and curved bodies have been noted in hatchlings of a wide variety of anurans in response to salt (Beebee, 1985; Chinathamby et al., 2006; Gosner and Black, 1957; Padhye and Ghate, 1992; Ruibal, 1959; Sanzo and Hecnar, 2006), as have abdominal cysts (edemas, "swollen bellies"). Shrunken heads (microcephaly) (Padhye and Ghate, 1992) and malformed gills (Ruibal, 1959) have also been noted in anuran embryos exposed to salt. Cysts and spinal deformities in response to salt have been described in the only caudate species studied to date, *Ambystoma maculatum* (Karraker and Ruthig, 2009). Thus, the deformities we describe in newt hatchlings appear to be diagnostic of the effects of salt.

Amphibian egg membranes are highly permeable and sensitive to osmotic changes in salt solutions (Hunter and De Luque, 1959). Road salt (NaCl) approximately equal to our "low" concentrations (~1 g/l Cl^-) has been shown to irreversibly disrupt the osmoregulatory ability of salamander eggs (Karraker and Gibbs, 2011). This disruption caused shrinkage of the perivitelline space (Karraker and Gibbs, 2011), substantially decreasing the area in which the embryo can develop, and has been the suggested cause of curved body and tail deformities seen in anuran hatchlings (Padhye and Ghate, 1992).

Physiological stress (increased glucocorticoids) has also been known to result in spinal deformities in fish (Eriksen et al., 2008) and could be partly responsible for the bent bodies and tails we observed in embryos developing in the physiologically stressful salt environments. It makes sense that other deformities we observed, such as large swellings and shrinkages of parts of the developing embryos' bodies are also due to osmoregulatory disruption of the developing embryo, through egg membrane degradation (Gosner and Black, 1957), and/or the inability of developing embryos to osmoregulate (Chinathamby et al., 2006).

We believe that ours is one of the first studies to take a quantitative approach in describing the frequency and severity of different developmental deformities in response to salt, as opposed to simply mentioning their occurrence. Attempting to quantify severity is important because not all deformities are equal in their effects on fitness. For example, acephalous hatchlings died within minutes of hatching, while hatchlings with only a small kink in their tail could survive indefinitely. Karraker (2007) found that most frog hatchlings with large cysts (scored as a severe deformity in our study) died within a week of hatching, and the occurrence of this deformity was strongly associated with salt concentration. Eggs developing in higher salt concentrations were significantly more likely to develop severe deformities, with the majority of the most severely deformed individuals in our study developing in medium and high salt concentrations. This gives us important information, often missing in other, more anecdotal studies, regarding the effects of these concentrations on amphibian fitness.

The contamination of amphibian breeding habitats with road deicing salts presents these animals with serious survival problems. Road salts cause eggs to die, and those that

survive often hatch out sooner, less well developed, at a smaller size, and more deformed (Gosner and Black, 1957; Hopkins et al., 2012a; Karraker, 2007; Karraker et al., 2008; Karraker and Ruthig, 2009; Padhye and Ghate, 1992; Ruibal, 1959). Nearly all studies on the effects of road salts on amphibians have focused only on anurans. However, the majority of these studies actually did not detect developmental deformities until salt concentrations were used that were far higher than those used in our study (i.e., 4.0-6.0 g/l vs. 2.0 g/l Cl^-) (e.g., Beebee, 1985; Chinathamby et al., 2006; Ruibal, 1959). This concurs with results found for *A. maculatum* (Karraker and Ruthig, 2009) that suggest caudates are more susceptible to salt than anurans. It may aid future research and management decisions to be aware of this apparent discrepancy in sensitivity among amphibian groups.

Our results also suggest that the emerging deicer, $MgCl_2$ appears to be overall as potent in its effects as the traditionally used deicer, NaCl. $MgCl_2$ has become the principal or sole deicer throughout much of the range of *T. granulosa*, and our research, reported here, and elsewhere (Hopkins et al., 2012a (Chapter 4)), indicates that its effects are severe on the embryonic development of this amphibian. This chemical deicer is currently the second most commonly used salt in North America (National Transportation Research Board, 2007), and Mg^{2+} has been found to be the most commonly found salt cation in roadside soils in parts of the continent (Cunningham et al., 2008). As the use of $MgCl_2$ increases, more studies should investigate the potentially severe effects of this environmental pollutant on a variety of organisms and life-history stages, to fully assess its ecological impact.

Conclusions

Road deicing salts, at environmentally relevant concentrations (Environment Canada, 2001) caused increased frequency and severity of developmental deformities in *T. granulosa* hatchlings. Eggs reared in control solution fared significantly better than eggs in any salt treatment, and increased salt treatment concentration resulted in increased severity of deformities. Results were similar for NaCl and $MgCl_2$, indicating that $MgCl_2$, an emerging stressor, does indeed cause developmental deformities in this amphibian, and at a similar rate as the more traditionally used NaCl. Quantification of the effects of this emerging pollutant on understudied species and life-history stages is critical to ensure the conservation of amphibian populations and biodiversity, especially given results such as ours and others (Karraker and Ruthig, 2009) suggesting that caudates may be more susceptible to salt than more frequently studied anurans.

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CHAPTER 6

DEVELOPMENTAL AND EVOLUTIONARY HISTORY AFFECT SURVIVAL IN STRESSFUL ENVIRONMENTS[5]

The world is increasingly impacted by a variety of stressors that have the potential to differentially influence life-history stages of organisms. Organisms have evolved to cope with some stressors, while with others they have little capacity. It is thus important to understand the effects of both developmental and evolutionary history on survival in stressful environments. We present evidence of the effects of both developmental and evolutionary history on survival of a freshwater vertebrate, the rough-skinned newt (*Taricha granulosa*) in an osmotically stressful environment. We compared the survival of larvae in either NaCl or $MgCl_2$ that were exposed to salinity either as larvae only or as embryos as well. Embryonic exposure to salinity led to greater mortality of newt larvae than larval exposure alone, and this reduced survival probability was strongly linked to the carry-over effect of stunted embryonic growth in salts. Larval survival was also dependent on the type of salt (NaCl or $MgCl_2$) the larvae were exposed to, and was lowest in $MgCl_2$, a widely-used chemical deicer that, unlike NaCl, amphibian larvae do not have an evolutionary history of regulating at high levels. Both developmental and evolutionary history are critical factors in determining survival in this stressful environment, a pattern that may have widespread implications for the survival of animals increasingly impacted by substances with which they have little evolutionary history.

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INTRODUCTION

Natural and anthropogenic stressors are commonplace throughout the environment. The ways in which stressors impact organisms, and their ability to successfully respond to these stressors is of paramount importance to our understanding of biological systems. For organisms with complex life cycles, the ability to respond to a given stressor may vary depending on life-history stage, and there may be carry-over effects from one stage to the next [1] (see Table S1 in Supporting Information). However, organisms may or may not have an evolutionary history of regulating the stressor in question, and this may also affect their ability to effectively respond [2,3]. We propose that both an organism's developmental history of exposure to a stressor (developmental history hypothesis) and its evolutionary history of regulating that stressor (evolutionary history hypothesis) play critical roles in the survival of organisms in stressful environments.

It has been suggested that the earlier in an organism's life-history environmental stressors are experienced, the more severe the lasting consequences will be [4-6], and there is strong empirical evidence across animal taxa for this assertion (Table S1). This forms the basis of our developmental history hypothesis. In humans, for example, the environment of the womb can significantly affect an individual's chances of cardiac and other diseases later in life [5,7,8]. In birds, the temperature at which eggs are incubated can affect hatchling body composition, growth, immunocompetence and thermoregulatory ability [4,9]. Developmental temperature also affects survival, growth and behavior of juvenile reptiles (e.g., [10]) (Table S1). Elevated CO_2 as embryos results

in decreased larval settlement success of sea urchins [11], and the ability of bryozoans to produce large, successful colonies is dependent on their embryonic experience and growth [12]. Thus, embryonic exposure to stressors can be critical to an animal's future fitness (Table S1).

Parsing critical life-history stages, however, is not trivial, and many studies have given contradictory evidence for the developmental history hypothesis. For example, while multiple studies have shown that embryonic environment can significantly affect an individual's chances of success in later life (Table S1), others have shown that it is the larval or juvenile environment that has the greatest influence on survival, growth, or reproduction (e.g., [13,14]). Still others have shown that while the embryonic environment has a significant role to play in later life, its effect may be dependent on the environment animals experience later in life (e.g., [15,16,17]). Experiments are often not designed to isolate the effects of environment on a specific life-history stage from those of another (e.g., [18,19-21]), and thus, consistent knowledge of the environmental and carry-over effects across multiple life-history stages is lacking (but see [14,17,22,23]).

While there is a strong empirical basis for the developmental history hypothesis (even with the conflicting evidence and limitations identified above), there is much less known regarding the evolutionary history hypothesis. Organisms in most habitats today face both natural stressors with which they have an evolutionary history, and thus evolved physiological mechanisms of regulating (e.g., CO_2, temperature, NaCl), and novel stressors with which they do not have this same evolutionary history (e.g., pesticides, flame retardants, commercial non-NaCl-based deicing salts), and thus lack the physiological mechanisms to regulate. The effects of developmental history must

therefore be placed in this environmental and evolutionary context. While many studies have documented the significant effects of unfamiliar substances such as pollutants on evolutionarily-naïve organisms (e.g., reviewed by [24] for amphibians), these cannot be directly compared to stressors with which the organism has an evolutionary history, and thus a means of regulating, as the nature of the two stressors is usually very different (i.e., comparing the effect of a herbicide with the effect of NaCl). At this point, we do not know how the potentially important effects of an organism's evolutionary history with a stressor may interact with its developmental history of exposure with the stressor.

To address these concerns, we tested the effects of developmental and evolutionary history on survival in stressful environments. We chose the rough-skinned newt (*Taricha granulosa* Skelton; Caudata: Salamandridae) as our model, an osmotically sensitive organism, and salinity as its stressor. Specifically, we tested the effects of both NaCl and $MgCl_2$ on the post-hatching survival of newt larvae that had either been exposed to salt as both embryos and larvae or just as larvae. Salinity is an excellent stressor to use to test our two hypotheses, as it is a naturally occurring abiotic component of aquatic habitats, and is known to have significant carry-over effects from the embryonic to post-hatching life stages in a variety of organisms, [15,23,25-27] (Table 6.3 (Supporting Information)). We used salt concentrations that were within environmentally relevant limits of freshwater aquatic systems impacted by either natural (i.e., estuaries) or anthropogenic (i.e., road deicing salts) sources of salts [29,30]. The two most common sources of salinity in North America today are two different salts, NaCl and $MgCl_2$, only one of which most organisms have an evolutionary history of regulating. Sodium chloride (NaCl) is one of the most common osmolytes, and organisms have an

evolutionary history of regulating this in a variety of habitats, whereas $MgCl_2$ has not been identified as a common vertebrate osmolyte [31], and Mg^{2+} is not found in substantial concentrations in most freshwater habitats, nor the precipitation that feeds them (including in the newts' range) [32]. Therefore, animals do not have the same evolutionary history of physiological regulation of this ion. Nevertheless, $MgCl_2$ is now the second most commonly used road deicer in North America (behind NaCl), and is used exclusively in some areas of the continent [33]. Thus, there is the potential that organisms will encounter $MgCl_2$ in substantial quantities in their environment. We found that both salts caused significant developmental carry-over effects from the embryonic environment on larval survival, but that the salts differed in their effects on larval survival, according to the differential evolutionary history that amphibians have with regulating the two stressors. As more and more freshwater animals, mostly maladapted to salt, will be forced to cope with increasing salinization of their habitats due to the application of road deicing salts [34,35-36], landscape modification and agricultural waste [37-40], and rising sea-levels [41-43], understanding the effects of both developmental and evolutionary history of salinity exposure will have important implications for both life-history and evolutionary theory, as well as conservation efforts.

MATERIALS AND METHODS

Ethics Statement

Adult rough-skinned newts (*Taricha granulosa*) (not an endangered or protected species) were collected by dip-net and hand from Soap Creek ponds (44˚40'13.22"N,

123˚16’39.65”W) under Oregon Department of Fish and Wildlife Scientific Taking Permit #062-11. Access to these ponds was granted by Joe Beatty, Oregon State University. The Utah State University Institutional Animal Care and Use Committee (IACUC) approved the collection and use of animals in this research, and all experimental protocols (approved protocol #1524). Animals were euthanized at the completion of experiments with MS-222, in accordance with the approved IACUC protocol (#1524).

Experimental Procedure

As reported in a previous study ([44] for detailed methods on habitat, field collection, rearing eggs and preparing salt solutions), we reared eggs from 16 different gravid wild-caught female rough-skinned newts (*Taricha granulosa*) from a single, salt-naïve population from Benton County, Oregon, in a laboratory environmental control chamber at 7˚C. This population is truly salt-naïve [44], being highly philopatric to freshwater ponds that are separated by hundreds of meters from small county roads that are not salted (Kendal Weeks, Oregon Department of Transportation Road Maintenance, personal communication; Kent Mahler, Benton County Road Maintenance, personally communication). While $MgCl_2$ is widely used in Oregon as its exclusive deicer, it is also not applied to the nearest stretch of highway to these ponds, located over 4km away (Kendal Weeks, Oregon Department of Transportation Road Maintenance, personal communication). See [44] for additional details on this habitat. Eggs from wild-caught females were randomized to one of six different salt treatments, made with laboratory grade NaCl (Thermo Fisher Scientific, Fair Lawn, NJ, USA), $MgCl_2$ (Acros Organics,

Fair Lawn, NJ, USA) and distilled water (Low NaCl, Low MgCl = 1.0 g/l Cl^-; Medium NaCl, Medium $MgCl_2$ = 1.5 g/l Cl^-; High NaCl, High $MgCl_2$ = 2.0 g/l Cl^-) and a control (20% Holtfreter's Solution = 0.7 g/l Cl^- [45]). Those eggs that survived these treatments were used in the present experiment. At hatching, the size (total length) and developmental stage [46] of hatchlings were recorded (see [44] for full methods and results).

Eggs that were reared in a salt treatment remained in that salt treatment as larvae (Fig. 6.1). Approximately 7 times more control eggs were reared than salt treatment eggs, so that control eggs could be randomized to new larval treatments in the present experiment (similarly to [26]) (Fig. 6.1). Eggs were monitored daily and all larvae were transferred to their new treatment solution within 12 hours of hatching. This direct transfer, following a similar protocol of Petranka and Doyle [47], was meant to mimic the sharp spike in Cl^- concentrations found in road-side environments that immediately occurs within hours of a deicing event or snowmelt [48-50], where minimal to no time is allowed for acclimation. While gradual acclimation of low salinity levels have led to increased tolerance in some amphibians (e.g., [51]) it has also led to increased susceptibility in others [52], and is less environmentally relevant to examining the sudden spikes of salinity seen in habitats due to road deicing salt application. In addition, while the salt concentrations used were typical for those immediately resulting from deicing events [48,53], they were also well below recorded NaCl and $MgCl_2$ LD-50 values for other amphibian larvae [37,54,55].

Larvae were housed in sibling groups of up to 5 individuals (keeping offspring from different female and treatment combinations separate) in 12.5 cm diameter, 10.5 cm

deep round plastic containers, filled with 400 ml of solution. Each container was randomized to a location in a growth chamber set at 7˚C, with a 12 h light : dark photoperiod. Containers were checked daily for larval survival, and dehydration. Dead larvae were noted and removed, and a small amount of distilled water was added to each container if necessary, to compensate for evaporation. *Taricha granulosa* larvae retain some embryonic yolk for up to approximately two weeks after hatching, and do not engage in feeding on prey before then. As we did not want to confound our survival results with possible negative effects of the salt treatments on larval prey, we only conducted this experiment for 15 days post-hatching; if a larva was alive at day fifteen, it was recorded as alive for the purposes of the analysis. A similar endpoint has also been used in a previous study on post-hatching survival of frog larvae in road deicing salt [47].

Statistical Analysis

For survival analyses, individual larvae were treated as subsamples within containers, which were treated as subsamples nested within individual female. Larval survival was analyzed using a binomial distribution, with a generalized linear mixed model blocking on individual female as a random effect. We first compared the survival of control newts (i.e., those reared in control as eggs and larvae) to newts in all other treatments for each salt, and then ran separate models to compare survival among salt treatments (minus control) for both larvae that were reared in control and those reared in salt as eggs, with Tukey-adjusted multiple comparisons among individual treatment levels, when an overall significant effect of treatment was found. We were, however, primarily interested in comparing and contrasting the effects of embryonic and larval

environment on larval survival. As we did not have a complete factorial design in this study (e.g. embryonic low $MgCl_2$ + larval high NaCl treatment combination), for this analysis, we analyzed the effects of the two different salt types separately, using embryonic and larval treatments as fixed effect factors in our models. We then analyzed the effect of embryonic versus larval environment on larval survival for each salt [56]. In these analyses, larval treatment had three levels, low, medium and high, and embryonic treatment had two levels, control and salt. This enabled a direct statistical comparison to be made of larval survival between animals that were reared as eggs in control or, for example, low $MgCl_2$, for larvae that were reared in low $MgCl_2$. We conducted Tukey-adjusted multiple comparisons, specifically comparing larval survival in each salt treatment level between eggs that were reared in either that salt treatment or control, for cases in which an overall significant effect of either embryonic treatment, larval treatment, or their interaction was found. Analyses were conducted using PROC GLIMMIX in SAS software version 9.3, with significance set at $\alpha = 0.05$.

As embryonic exposure to salt affected the size and developmental stage at hatching of newts, as did differences among individual mothers (females) [44], we wanted to further assess the potential contribution of these variables, as well as embryonic and larval treatments in general, in explaining any overall effects of salt treatment in either embryonic or larval environments on larval survival. To do this, we conducted multivariate classification analyses, which measure variable importance in a model's ability to correctly classify larvae as having died or survived. As only one out of 778 newt larvae died after being reared in control as both an embryo and larvae (see Results), we restricted our analyses to larvae reared in salt post-hatching. We used three

validated classification procedures [57], logistic regression, Classification Trees [58] and Random Forests [57,59], and in each case assessed variable importance by examining the relative classification performance of models incorporating or not incorporating key variables.

Specifically, we assessed the ability of the models to correctly classify larvae as having died (sensitivity). For the full model, we included all larval and embryonic variables of potential interest, including: larval treatment, embryonic treatment, length at hatching, developmental stage at hatching, and female identity. We then withdrew the larval treatment variable, and reassessed the model's sensitivity, withdrew all embryonic variables (leaving only larval treatment and individual female) and again reassessed the model's sensitivity, to assess the potential relative contribution of larval environment in predicting larval mortality. As well as assessing variable importance in this manner, all three classification methods also provide separate indicators of variable importance [57]. This is achieved through a variable importance plot in Random Forests, a classification plot in Classification Trees, and the variable with the largest Wald Chi-Square value in logistic regression. We chose the most important variable identified in each of these methods from the original full model, and reinserted it back into the model including only larval treatment and female identity, and assessed whether the inclusion of this identified variable increased model performance. Classification analyses were completed in SAS (logistic regression) and R (R Development Core Team, 2008, www.R-project.org) (Classification Trees and Random Forests). Finally, as length at hatching was identified as a key variable of importance in predicting larval mortality (see Results), we compared

the mean length at hatching of larvae that died versus survived in each treatment using t-tests in SAS software version 9.3, with significance set at $\alpha = 0.05$.

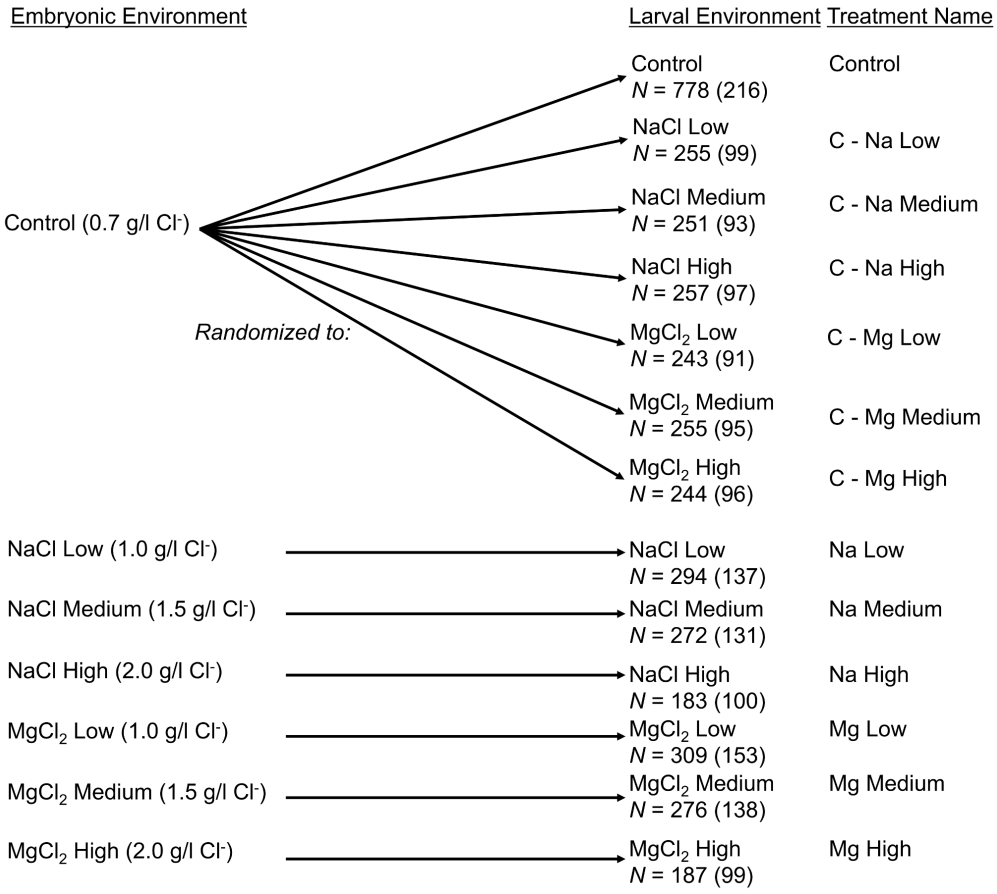

Figure 6.1. Outline of experimental design. Embryonic and larval environments, salinity concentrations, treatment names, and sample sizes are shown. Newt eggs were reared in either a freshwater control, or one of six salt treatments. Upon hatching, embryos that were reared in salt stayed in that salt, whereas embryos reared in control either stayed in control or were randomized to one of the six salt treatments for the larval environment. The name of each treatment combination is listed, and sample sizes are given under each larval environment (numbers outside of parentheses indicate total number of individuals in the treatment, whereas numbers inside parentheses indicate number of containers in the treatment (up to five sibling larvae were reared in the same container and individuals within containers were treated as nested subsamples. See Methods for more details).

RESULTS

After 14 days, only one out of 778 larvae reared in control as both egg and larva ("control" treatment) died in this treatment, which was significantly fewer than in any other treatment (all $p<0.001$). The survival of the remaining larvae, all experiencing salts in their larval environment, was then compared. There was a significant effect of larval salt treatment on larval survival for both newts that were reared embryonically in salt ($F_{5,74}=16.54$, $p<0.0001$) and control ($F_{5,73}=7.81$, $p<0.0001$). For animals that were reared as eggs in salt and stayed in that salt as larvae, significantly more larvae died in low and medium $MgCl_2$ than in those corresponding concentrations of NaCl (all Tukey adjusted multiple comparisons $p<0.0001$), with a similar percentage of larvae dying in high $MgCl_2$ as high NaCl (Tukey adjusted $p=0.98$). For animals that were reared as eggs in control and then transferred to salt as larvae, marginally more larvae died in low $MgCl_2$ than low NaCl (Tukey adjusted $p=0.0698$), and significantly more larvae died in medium and high $MgCl_2$ than the corresponding concentrations of NaCl (Tukey adjusted $p<0.02$).

Increased salt concentration, in both the embryonic and larval environments, generally resulted in increased larval mortality (with the exception of high $MgCl_2$) (Fig. 6.2). For both salts, larval survival was significantly affected by embryonic environment (Table 6.1). For the majority of treatment levels, larvae that were reared as eggs in control solution survived significantly better than larvae that were reared as eggs in salt treatments (Fig. 6.2). For NaCl, both embryonic and larval treatments significantly affected survival of larvae in this salt, but for $MgCl_2$, only embryonic treatment

significantly explained larval survival (Table 6.1). There were no significant interacting effects of embryonic and larval environments on larval survival (Table 6.1).

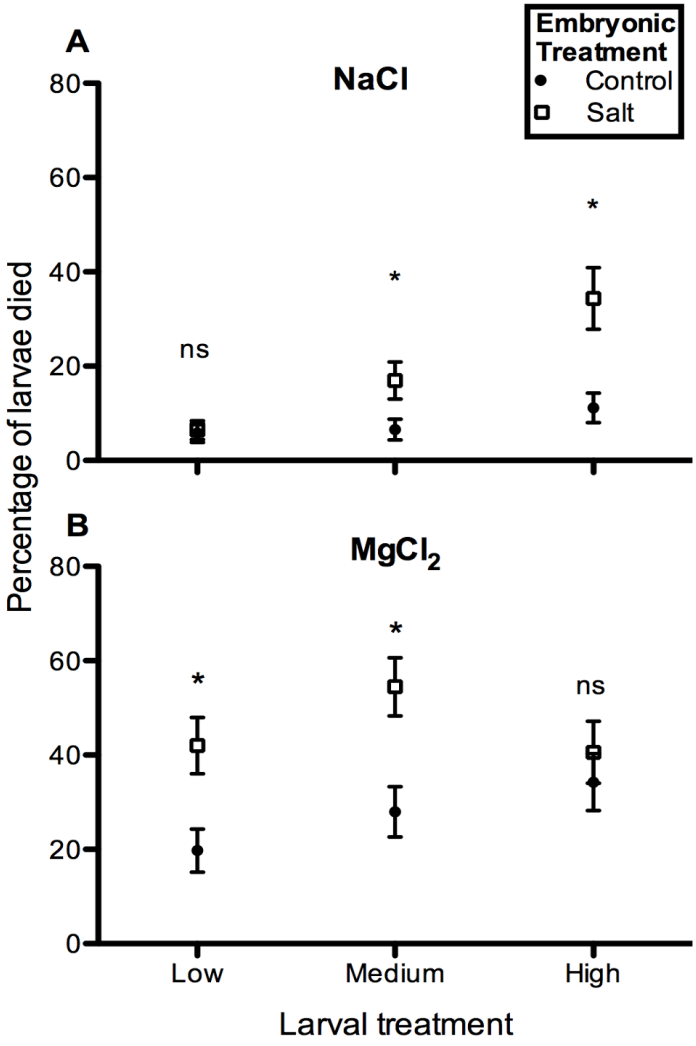

Figure 6.2. Percentage (mean ± SE) of larvae that died in each salt treatment. (A) NaCl, (B) $MgCl_2$. Only 1 out of 778 larvae in Control died, and thus only results for mortality in salt treatments are shown. Direct comparisons are made between the mortality of larvae reared as embryos in salt (open squares) or control (closed circles). Asterisks indicate significant differences (Tukey-adjusted multiple comparisons) between the percentages of larvae died in each of these treatments (i.e., for the larval treatment Medium NaCl, significantly more larvae died when reared as eggs in that salt, than did larvae reared as eggs in control). "ns" = no significant difference between treatments.

Table 6.1. Effects of embryonic environment, larval environment, and their interaction on larval survival in NaCl and $MgCl_2$. Significant effects are listed in bold.

Salt type	Embryonic environment			Larval environment			Embryonic x Larval environments		
	F	df (n,d)	*p*	*F*	df (n,d)	*p*	*F*	df (n,d)	*p*
NaCl	11.19	1,74	**0.0013**	9.61	2,74	**0.0002**	2.28	2,74	0.1095
$MgCl_2$	18.34	1,73	**<0.0001**	2.22	2,73	0.1162	2.00	2,73	0.1429

Eggs that were reared in salt water resulted in smaller larvae at hatching than those reared in control [44]. Classification analyses with three different methods all revealed length at hatching as the consistently most important variable in determining larval survival (Table 6.2), further strengthening the evidence of the importance of embryonic environment on survival post-hatching. Although Classification Trees and Random Forests had better sensitivity than logistic regression (as was expected [57]), the ability of models, using any of the classification methods, to correctly classify larvae as having died declined dramatically with the exclusion of embryonic variables (i.e., larval treatment and female identity alone was a very poor classifier of larval survival), but recovered substantially with the re-inclusion of length at hatching as a predictor variable (Table 6.2), further identifying it as a critical variable for predicting larval survival. Larvae that survived, in each of the treatments, were significantly larger at hatching, on average, than larvae that died (Fig. 6.3; for all t-tests, $p<0.01$).

Table 6.2. Classification analyses for predicting whether or not newt larvae died ("sensitivity"), for data excluding control data (i.e., only newts in salt as larvae). Three multivariate classification methods were utilized (logistic regression, Classification Trees, and Random Forests) to determine the most important variables predicting larval survival in salt. See Methods and Results for more details regarding these analyses and their interpretation.

Model	Classification method	Model sensitivity (%) (percent larvae correctly classified as having died)	Change in model sensitivity from full model sensitivity (%)	Most important variable identified
Full (Larval Treatment, Egg Treatment, Length & Stage at Hatching, Female)	Logistic Regression	47.70	.	Length at Hatching
	Classification Trees	69.65	.	Length at Hatching
	Random Forests	64.78	.	Length at Hatching
Just Embryonic Variables (Egg Treatment, Length & Stage at Hatching, Female) (not Larval Treatment)	Logistic Regression	39.82	-7.88	Egg Treatment
	Classification Trees	64.39	-5.26	Length at Hatching
	Random Forests	59.26	-5.52	Length at Hatching
Just Larval Treatment and Female	Logistic Regression	13.67	-34.03	Larval Treatment
	Classification Trees	19.84	-49.81	Larval Treatment
	Random Forests	14.32	-50.46	Larval Treatment
Just Larval Treatment, Female, & Length at Hatching	Logistic Regression	44.42	-3.28	Length at Hatching
	Classification Trees	61.76	-7.89	Length at Hatching
	Random Forests	52.56	-12.22	Length at Hatching

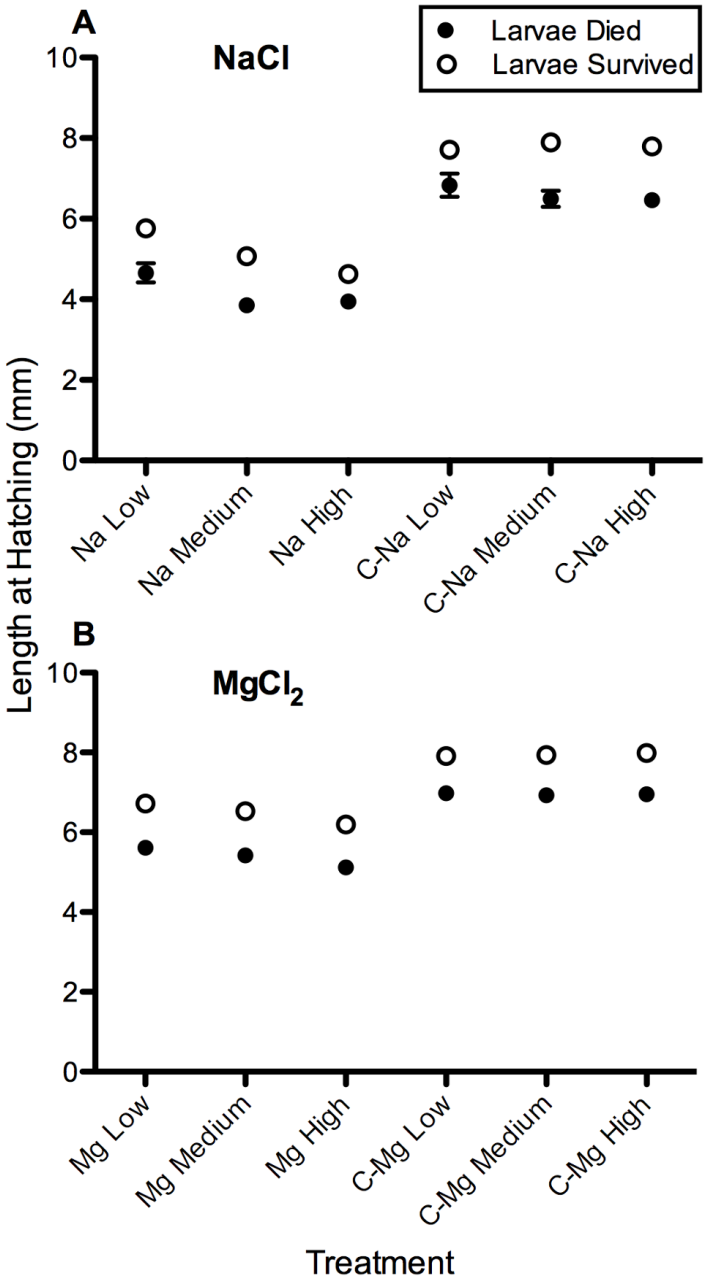

Figure 6.3. Mean (±SE) lengths at hatching (mm) of larvae that died (closed circles) or survived (open circles) in each salt treatment. (A) NaCl, (B) $MgCl_2$. In all treatments, larvae that survived averaged larger at hatching than those that died (all t-tests, $p<0.01$). See [44] for full results on length at hatching.

DISCUSSION

Developmental and evolutionary history each significantly affected the survival of newt larvae in salts, and thus the importance of both hypotheses was supported. Eggs appear to be a critical life-history stage for this amphibian in osmotically stressful environments. Animals that were exposed to salt as embryos and survived hatched at a smaller size than animals that did not experience embryonic salinity. Stunting of embryonic growth put amphibian larvae at greater risk for salt-induced mortality (Table 2). However, our results show that it is also important to understand the evolutionary history an organism has with a stressor. Even though there was no difference in egg mortality between embryos reared in NaCl or $MgCl_2$ [44], more larvae died in $MgCl_2$ than in NaCl (Fig. 4). While newt larvae have evolved with natural sources of NaCl in their environment, which they can osmoregulate, such common regulation of $MgCl_2$ does not appear to have evolved. Understanding this evolutionary history, as well as parsing critical life-history stages is imperative to understand the effects of stressors on the life-history of an organism.

The majority of organisms have complex life cycles, and the experiences of one life stage can have profound impacts on those in subsequent stages [1] (Table 6.3 (Supporting Information)). Embryonic salinity is known to affect the post-hatching survival, growth and development of marine and estuarine invertebrates, such as barnacles [23], crabs [15,25,27], horseshoe crabs [26] and tunicates [28]. While all life-history stages of amphibians have, individually, repeatedly been found to be extremely sensitive to salt [21,47,54,55,60-68], with a few notable exceptions such as *Fejervarya*

cancrivora [69,70], the relative sensitivity of each life-history stage, and potential down-stream effects of salinity from one stage to the next, have been less studied. In one of the only other studies on amphibians to examine embryonic carry-over effects of salinity, frog larvae (*Lithobates sylvaticus*) reared in salt water (NaCl-based) as eggs had reduced survival in salt compared to larvae that were reared in freshwater as eggs [47]. This study also found that growth and development of larvae that survived was depressed in those animals reared embryonically in salt, also suggesting carry-over effects of embryonic exposure to salt [47]. Snodgrass et al. [21] also found that *Bufo americanus* toadlets exposed to stormwater pond sediment (which had an increased conductivity mainly due to road deicing salt) as embryos were smaller at metamorphosis than embryos and larvae exposed to freshwater (although the relative effects of embryonic vs. larval exposure were not separated). Other studies have also shown potential carry-over effects of larval salinity exposure on metamorphic traits important for adult fitness [37,60]. These results all clearly show that Qiu and Qian's [23] statement regarding marine invertebrates, that "osmotic stress experienced in one life-stage can be passed over to the next life-stage," can apply to freshwater vertebrates as well.

This pattern of decreased post-hatching survival as a consequence of embryonic exposure has also been found in amphibians in response to other stressors, such as nitrite [71] and pesticides [22]. Thus, studies that do not examine effects at each life-history stage and do not consider the potential for cascading effects across stages may seriously underestimate the cumulative effects of exposure to stressors [19,71-73].

One of the primary ways that osmotic stress affects the embryonic stage to influence post-hatching survival is through the retardation of growth and development.

Newt eggs that were reared in salt water hatched sooner, smaller and less developed than newts reared in a freshwater control [44], and this resulting reduced length at hatching appears to be the single most important variable in predicting next-stage (larval) survival in salt water (Table 6.2). Size at hatching/birth is well known to have important implications on larval, juvenile, and adult health and survival in a wide variety of taxa, ranging from sea snails [74] and bryozoans [12], to birds [75] and humans [7]. Furthermore, this link between size and fitness has been identified as key to life-history theory [12]. Among amphibians, hatching early, smaller and less developed, is known to affect larval survival, the onset of feeding competence, competitive and predatory interactions, and larval growth rate and timing of metamorphosis [6,76-80]. Similar to our findings, small, less developed amphibian larvae are more susceptible to pollutants than are large larvae [81,82]. Smaller larval rough-skinned newts are also more vulnerable to be injured and die in predatory encounters with dragonfly nymphs [83]. Thus, even if smaller hatchlings are able to survive short-term in osmotically stressful environments (which seems unlikely from our results (Fig. 6.3)), or even if compensatory growth occurred later in development, a host of other fitness consequences of this initial stunted embryonic growth and development are still likely later in life [84], further emphasizing the importance of the embryonic environment for life-time fitness.

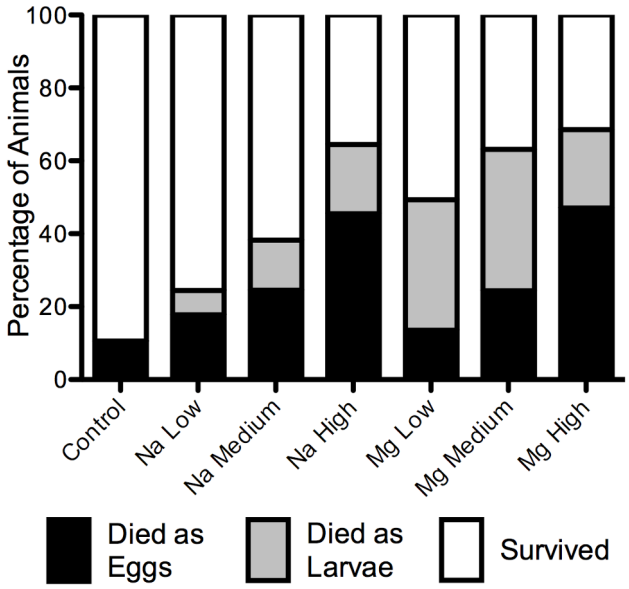

Figure 6.4. Mortality of newt eggs (black bars) and larvae (grey bars) in each salt treatment. This figure shows only larvae that were reared as both eggs and larvae in salt. The percentage of individuals that survived in each treatment is indicated in white. All percentages are calculated based on the total number of eggs that started in each treatment (Control = 2577, low NaCl = 363, medium NaCl = 366, high NaCl = 345, low $MgCl_2$ = 369, medium $MgCl_2$ = 369, high $MgCl_2$ = 354; [44]), some of which either died (black bars), or survived to hatching and were reared in salt, where they either died (grey bars) or survived (white bars).

While the effects of the two salt types were not significantly different on embryonic survival [44], there were differences in the larval stage, whereby $MgCl_2$ had relatively greater effects on survival (Fig. 6.4). This is in spite of the fact that embryos actually hatched slightly larger at $MgCl_2$ than at NaCl [44]. Although most amphibian eggs, like those of many other aquatic organisms [85], have little means of osmoregulating at the salt concentrations used in this study [37,63,86], and thus the effects of NaCl and $MgCl_2$ at this life-history stage are equally destructive (any effect of

evolutionary history is minimized in the absence of regulatory ability), amphibian larvae have evolved to osmoregulate Na^+ and Cl^- ion concentrations in their body through the use of integumental and gill Na^+ pumps [31,87-90]. Larvae have not evolved this same ability to regulate Mg^{2+} ions, however, and thus larvae in NaCl were able to attempt osmoregulation to survive in this solution whereas larvae in $MgCl_2$ were not. In addition to lacking this evolutionary history of osmoregulation, Mg^{2+} has also been shown to be inhibitory to important osmoregulatory skin ion pump functioning in other amphibian larvae [91]. Whereas the effects of NaCl on larvae act in a typical dose-response fashion (Fig. 6.2a), it appears that any concentration of $MgCl_2$ is detrimental to larvae (Fig. 6.2b), as they have less means to regulate it. This may explain why larval salinity concentration significantly influences larval survival for animals in NaCl, but not those in $MgCl_2$ (Table 6.1). The fact that Mg High had lower mortality than Mg Low or Medium (Fig. 6.2b) may be due to a number of possible reasons, including hormesis [92]. In the only other studies on the effects of $MgCl_2$ on amphibian larvae to date, both Dougherty and Smith [62] and Harless et al. [54] also found that this emerging deicing salt was more toxic to frog tadpoles than NaCl. Magnesium chloride may in fact, be more toxic than NaCl to life in general, as studies have found that otherwise salt-tolerant plants [93,94] and archaea [95] are often intolerant of $MgCl_2$, and the threshold for biological processes in $MgCl_2$ is lower than other salts, including NaCl [95]. These results make sense from an evolutionary perspective, given the small quantities of Mg^{2+} generally found in most aquatic ecosystems, relative to the higher quantities of Na^+ found in precipitation and the ocean [32], and thus many organisms may not have an evolutionary history of regulating Mg^{2+} in high concentrations in their environment.

Vulnerability of a particular life-history stage can be described as the ability of that life-history stage to regulate the stressor in question. Using this criterion, it appears that eggs are the most vulnerable life-history stage to salts overall in amphibians (this study; [61,65,66,96]) and effects on embryonic development at this stage have profound survival consequences in later life-history stages, even possibly affecting population viability indirectly through influencing post-embryonic (larval) mortality [97]. Similarly, amphibian larvae cannot successfully osmoregulate in $MgCl_2$, and thus all life-history stages are particularly vulnerable to this evolutionarily novel but emerging deicing agent, which is now the second most commonly used road deicer in North America [33].

Conclusions

Understanding the evolutionary history of an organism with its stressor, and the differential sensitivity of life-history stages to that stressor are critical in assessing the vulnerability of organisms to stressful environments. It is now apparent that embryonic exposure to a stressor can have profound implications on the post-hatching survival and fitness of organisms in practically all animal taxa (Table 6.3 (Supporting Information)), through influencing growth and development in this critical life-history stage. In post-hatching individuals, however, even the largest, best-developed organism can only successfully deal with stressors that they have evolved to regulate. As the world of these organisms becomes increasingly impacted by anthropogenic factors, understanding this evolutionary history and its survival implications at and across different life-history stages will be critical for the future conservation of animals in increasingly stressful environments.

Data Accessibility

Raw experimental data are publicly available at Dryad doi:10.5061/dryad.jg0j5

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SUPPORTING INFORMATION

Table 6.3 (S1). Animal phyla where components of the embryonic environment have been demonstrated to have significant carry-over effects post-hatching. This list is not exhaustive, but is representative of the diversity and breadth of this phenomenon throughout the animal kingdom.

Phyla Subphyla: Class	Examples	Traits / endpoints affected by embryonic environment	Component of embryonic environment	References
Bryozoa				
Gymnolaemata	Bryozoans	Larval size, leading to larval survival, growth rate, timing and rate of reproduction, size of subsequent embryos and larvae	Colony size	[1]
Arthropoda				
Crustacea : Maxillopeda	Barnacles	Larval survival; Time to metamorphosis	Salinity	[2]
Crustacea : Malacostraca	Crabs	Larval survival, body nutritional content, development, growth, starvation tolerance; size at metamorphosis; juvenile size; larval osmoregulatory ability;	Salinity; Temperature	[3-5, reviewed by 6]
Hexapoda : Insecta	Butterflies, Beetles	Larval size, energy content, body nutritional composition; size at hatching; adult size	Temperature	[7, 8]
Chelicerata : Merostomata	Horshoe Crabs	Length of larval period (molting to first juvenile instar)	Salinity	[9]
Mollusca				
Gastropoda	Sea Snail	Hatchling size, leading	Brood size	[10]

		to juvenile and adult survival, growth, time to maturity		
	Freshwater Snail	Juvenile predator avoidance behaviour	Temperature	[11]
Echinodermata				
Asterozoa	Seastar	Larval survival; larval growth; successful metamorphosis	Maternal nutrition	[12]
Echinozoa	Sea urchins	Larval survival; larval growth; successful metamorphosis; larval settlement success	Maternal nutrition; CO_2 acidity	[12, 13]
Chordata				
Urochordata : Ascidiacea	Tunicates (sea-squirts)	Adult functional development	Salinity; Temperature	[14]
Vertebrata : Actinopterygii	Ray-finned bony fishes	Juvenile body size and shape; larval prey-capture ability; larval growth rate and development; juvenile growth rate; larval and juvenile muscle growth and development; larval gut development; Survival up to 2 years post-hatching; Adult cardiac morphology, aerobic capacity (swimming performance); juvenile antipredator / predator avoidance behaviour; 1 year post-hatching humoral immunosuppression; larval survival	Temperature; Methylmercury; Acidity; Crude oil; Cadmium; Hypoxia; Pesticide (DDT) metabolites; Aluminum	[15-26]
Vertebrata : Amphibia	Frogs, toads, salamanders, newts	Larval survival; predator avoidance behaviour; predation survival; larval growth; larval insecticide susceptibility; larval feeding rate; larval size and development 1	Salinity; Stormwater sediments (increased conductivity); Temperature; UV-B radiation; Pesticide; PCBs;	This study, [27-48]

		month post-hatching; Size at metamorphosis; Juvenile behaviour and desiccation risk up to 8 months post-metamorphosis; Juvenile survival; Time to metamorphosis; swimming performance; size-independent morphology; larval activity level; Post-metamorphic (juvenile) morphology	Herbicide; Nitrite; Acidity (pH); Hydration; Conspecific chemical alarm cues; Predatory chemical cues; Predator presence;	
Vertebrata : Sauropsida ("Reptilia")	Turtles, Lizards, Snakes, Crocodilians	Hatchling size; Juvenile growth rate; Size at up to 2 years post-hatching; Sex; Escape behaviour; Juvenile survival up to 2 years; Juvenile habitat temperature choice and thermoregulation; Sprint speed; Juvenile activity level, ability to move, climb, avoid predators, shedding time and drinking speed; antipredator behaviour; body contraction strength; swimming speed	Temperature; Herbicide; Hypoxia	[49-57]
Vertebrata : Sauropsida ("Aves")	Birds	Juvenile growth, body condition, immunocompetence, metabolic costs of thermoregulation; Recruitment probability; Fecundity; juvenile morphology;	Temperature; Maternal Nutrition	[58-63]
Vertebrata : Mammalia	Rats, Humans, Moose, Hamsters	Sedentary behaviour; risk of cardiac disease; type 2 diabetes; adult body mass; adult offspring endocrine profiles and antipredator behaviour	Physical size of womb; Maternal nutrition; Climatic conditions (temperature, snow depth); Maternal immune activation	[63-68]

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CHAPTER 7

TIDAL NEWTS: PHYSIOLOGICAL RESPONSES TO AN OSMOTICALLY STRESSFUL ENVIRONMENT[6]

1. Organisms are increasingly exposed to environments with unpredictable, transient stressors, which provide physiological challenges to homeostasis. Coastal freshwater environments are a prime example of this phenomenon, where storms and sea-level rise lead to influxes of salinity, and osmotically challenge organisms.

2. Amphibians are particularly vulnerable to osmotic stress and have often been discounted from tidal environments as a result. Here, we report a large breeding population of rough-skinned newts (*Taricha granulosa*) inhabiting the tidal area of a stream on the Pacific coast of North America.

3. We compared physiological responses to salinity stress of newts from the unpredictable tidal area of the stream to animals from a stable upstream freshwater area in a common-garden laboratory environment.

4. Although elevated salinity significantly affected the osmotic (body weight, plasma osmolality, and Na^+/K^+-ATPase), stress (corticosterone), and immune (bactericidal ability) responses of newts, animals inhabiting the tidal area were generally less reactive to salt stress than those from the freshwater population upstream.

5. Our results provide possible evidence for some physiological adaptation in this species to this osmotically stressful environment. As environments become increasingly stressful

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and unpredictable, such studies will become increasingly important to our understanding of the abilities of organisms to physiologically respond, adapt, and ultimately survive.

INTRODUCTION

Few environments are fully predictable and free of stressors (Wingfield & Kitaysky 2002). Organisms are often exposed to stressors that cause deviations from homeostasis, and may initiate a physiological stress response (French, Matt & Moore 2006; Wingfield 2013). Yet, environments are also not uniformly stressful, and may be relatively stable for extended periods of time before a short-term, unpredictable stressful event occurs (Badyaev 2005; Bell 2013). In coastal environments, otherwise freshwater habitats are increasingly subjected to salinity due to a combination of stochastic storm events (Gunzburger et al. 2010; Trenberth 2011), and rising sea levels (Nicholls, Hoozemans & Marchand 1999; Lowe & Gregory 2005; Purcell et al. 2008). The temporary increase in salinity caused by these events is physiologically challenging for freshwater-adapted organisms (Purcell et al. 2008; Wu, Gomez-Mestre & Kam 2012).

Amphibians are particularly sensitive to salt stress due to their highly permeable skin and eggs (Shoemaker & Nagy 1977). As such, the presence of amphibians in coastal environments that are influenced by sea water has traditionally been discounted, despite existing knowledge of select amphibians capable of tolerating saline habitats (e.g., Neill 1958; Gordon, Schmidt-Nielsen & Kelly 1961; Gordon 1962; Katz 1989; Gomez-Mestre & Tejedo 2003). A recent review (Hopkins & Brodie 2015) reported that amphibians

inhabiting saline environments are not as rare as previously thought, although information on how most species achieve physiological tolerance of these environments is lacking. Understanding the physiological limits and adaptations of amphibians in coastal habitats is crucial, as amphibians living in these environments are increasingly being forced to cope with salinization brought on by events such as storm-surge and hurricanes (Thirion 2002; Gunzburger et al. 2010; Brown & Walls 2013). It has been hypothesized that populations in these dynamic environments may have to evolve tolerance to increased salinity in order to survive (Gunzburger et al. 2010; Moreira, Knauth & Maltchik 2015). Classical work on amphibian osmoregulatory physiology has elucidated physiological mechanisms based primarily on cutaneous exchange of water and Na^+ ions and the hypersynthesis and retention of urea in response to salinity for some species in relatively predictable estuarine environments (e.g., the crab-eating frog (*Fejervarya cancrivora*) in mangrove swamps) (e.g., Gordon et al. 1961; Shoemaker, Hillman, Hillyard et al. 1992; reviewed in Katz 2015). More recent work has suggested that the majority of saline habitats inhabited by amphibians are much more unpredictable and transitory in their salinity (Wu et al. 2012; Kearney et al. 2014). The physiological effects of transient salinity may be different compared to more predictable, constant exposure to salt (Kearney et al. 2014), and little work has been completed on the physiological responses to salt water of amphibians inhabiting these environments.

While classic osmoregulatory physiology work involving weight changes and plasma osmolality have been conducted on many amphibian species, increased salinity may also disrupt other physiological processes that are critical for fitness, including endocrine and immune responses. For example, in response to a stressor, the vertebrate

hypothalamic-pituitary-adrenal (HPA) axis initiates a hormonal cascade, resulting in the production of adrenal steroid hormones (glucocorticoids) (Sapolsky 1992). The production of glucocorticoids (corticosterone or cortisol depending on the species) is thus considered a general indicator of physiological stress (Sapolsky 1992; Moore & Jessop 2003). In addition to measuring glucocorticoids, there has been a recent push to assay functional physiological responses such as immunity (Hopkins & DuRant 2011; Breuner, Delhanty & Boonstra 2013). Assaying the bactericidal ability of blood plasma immune components (French & Neuman-Lee 2012) can yield important data on the effects of a stressor on components of immune function critical for fitness (Lochmiller & Deerenberg 2000). Finally, while studies involving the effects of stressors on physiology have often focused on either naïve or impacted populations, studies that directly compare a variety of physiological responses of individuals from each population type are less common but arguably more valuable (Hopkins & DuRant 2011).

In this paper we report the discovery of a large breeding population of rough-skinned newts (*Taricha granulosa* Skilton, Caudata: Salamandridae), a common caudate amphibian found along the Pacific coast of North America (Nussbaum, Brodie & Storm 1983), inhabiting an osmotically variable coastal tidal habitat, where dramatic storm events unpredictably wash sea-water upstream into otherwise fresh breeding habitats (Fig. 7.1). Ferguson (1956) anecdotally mentioned finding a few individual rough-skinned newts in the tidal area of a stream and beach on the southern Oregon coast, and the evolutionary potential for salinity adaptation in an inland population of this species has recently been documented (Hopkins, French & Brodie 2013). Here we expand these observations and investigate this potential by documenting tidal habitat use in this species

and comparing the physiological response to increased salinity in tidal newts to animals found further upstream in an osmotically-stable freshwater area. We performed multiple laboratory experiments to examine the physiological responses of newts from both habitats to salinity in a common-garden environment. We started with a classical metric for osmoregulatory physiology, change in body weight, and compared the responses of newts to other amphibians found in saline habitats. We followed this with in-depth analyses of osmotic (Na^+/K^+-ATPase and plasma osmolality), stress (release of corticosterone), and immune (bactericidal ability of blood immune components) physiology, comparing populations. We hypothesized that newts from the tidal area of the stream would be more resilient to short-term salt stress, and thus show fewer changes from homeostasis, than newts from the more stable freshwater area of the stream.

MATERIAL AND METHODS

Field Work

We surveyed tidal/estuarine streams on the southern Oregon coast, USA, in May 2012. Newts were found in the tidal area of Hunter Creek, a small estuarine stream in Curry County, Oregon, USA (42˚23'19.60"N, 124˚25'21.54"W; Fig. 7.1). We defined the tidal area of Hunter Creek as the area of the stream showing a visible tidal influence, including the presence of oceanic waves travelling upstream, and a substantial change in water level (at least 0.5 m) between high and low tides. The tidal area ranged from bare rock and sand near the mouth to littoral vegetation and shrubs on the banks of the stream (starting at ~ 350 m from the ocean). All measurements of distance from ocean are given

as distances at high tide in May 2012. There was ~200 m difference in the distance to the ocean at high vs. low tide in this estuary in May 2012. The tidal area of Hunter Creek is, like all estuaries (Vernberg & Vernberg 2001), extraordinarily dynamic, and distances from the ocean change dramatically over time, due to highly unpredictable weather events, including storms, rainfall, snowmelt, and wind (Fig. 7.1cd).

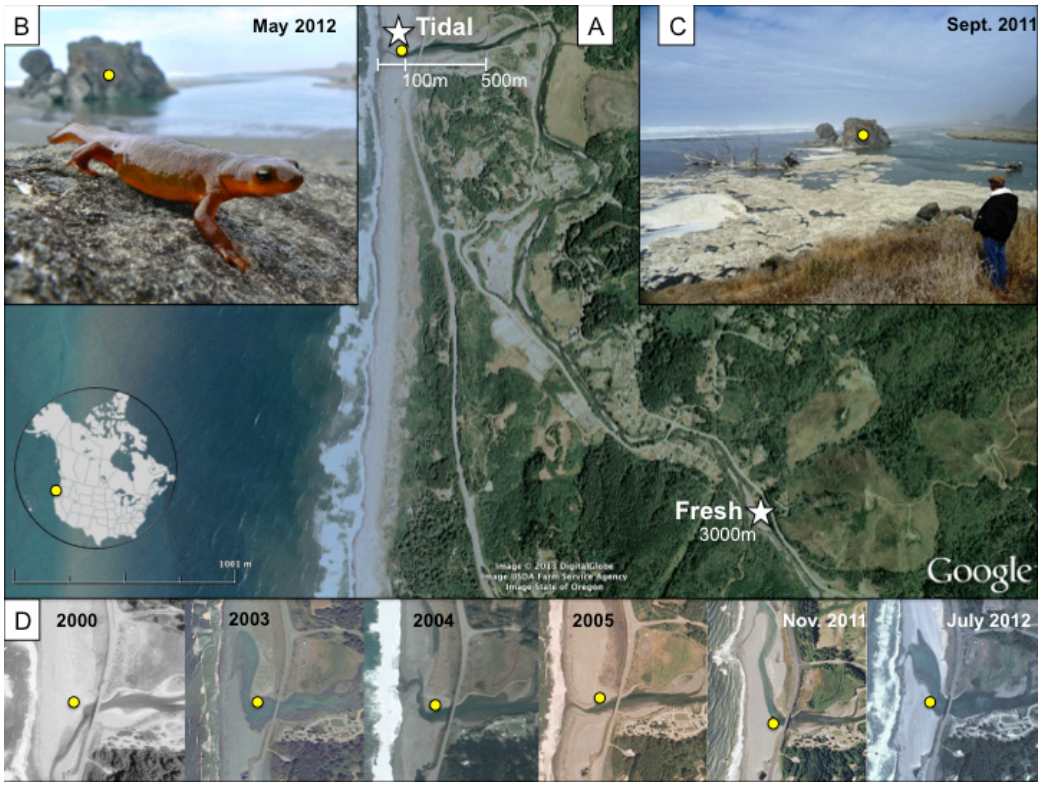

Fig. 7.1. (a) The study site, Hunter Creek, Oregon, USA, showing the location of tidal (a,b) and fresh (a) areas of the stream. Distances are measured from the Pacific Ocean at high tide in May 2012. Rough-skinned newts (*Taricha granulosa*) were located in the tidal area 100-500 m from the ocean (a,b). Yellow dots throughout the figure reference the position of Turtle Rock, the location closest to the ocean (100 m) where newts were found. (c,d) Demonstrates the dynamic nature of the tidal area from month to month and year to year, as the position of the stream on the beach changes dramatically relative to Turtle Rock. (c) A storm event where seawater washed completely around Turtle Rock and up the stream (white foam from the ocean is visible). Aerial photographs (a,d) from Google Earth™. Photograph in (c) by Rose Muenker, used with permission.

We surveyed the tidal area for newts, extending 500 m upstream from the ocean (Fig. 7.1a), noted the location and breeding condition (Nussbaum et al. 1983) of each animal found, and measured its snout-vent length, total length (includes tail), and mass, to ensure that a healthy breeding population was present in this area, and the newts we collected were not merely accidental transplants to the tidal habitat. The freshwater area of Hunter Creek (~3 km upstream) had no quantifiable tidal influence, with a lack of oceanic waves and no change in water level during tides. We surveyed this habitat for newts in the same manner as in the tidal area. Temperature and salinity environmental measurements were also taken in both habitats (detailed methods and results found in Supporting Information).

Laboratory Animal Care

Adult newts were taken back to Utah State University (Logan, UT, USA) and used in accordance with Utah State University Institutional Animal Care and Use Committee (IACUC) regulations (protocol #1524). Newts were individually housed in plastic containers (35 x 20 x 13 cm) with 2.0 L of filtered tap water and a styrofoam perch in a temperature control room set at 5˚C with a 12:12h light-dark setting. Newts were fed blackworms (*Lumbriculus variegatus*) *ad libitum* until used for experiments, 6-9 months later.

Laboratory Salt Challenge 1: Weight Change Experiment

Female and male newts from each population were transferred to a chamber set at 14˚C (average water temperature in Hunter Creek recorded in the field) in November

2012, and given 10 days to acclimate. Eight days prior to the beginning of the experiment, each newt was fed ~2 g of blackworms, and then fasted until the experiment began. Four to six male and four to six female newts from each population were randomly assigned to a salt solution of either 5 ppt or 10 ppt made with lab-grade NaCl (Mallinckrodt Baker, Inc., Paris, USA) and filtered tap water, or a control (0.2 ppt – filtered tap water). Thus, there were a total of 9-11 newts in each treatment. Salt concentrations were selected based on the general tolerances of freshwater (0-1 ppt), estuarine (4-8 ppt), and marine (10+ ppt) organisms (Vernberg & Vernberg 2001), and because 10 ppt has been proposed as a general upper level of salt tolerance for amphibians found in saline habitats (Gomez-Mestre & Tejedo 2003; Hopkins & Brodie 2015). On the morning of the experiment, newts were removed from their holding containers, patted dry with a paper towel, and weighed on a digital mass balance (±0.1 g). They were then immediately transferred to a container with 1.5 L of randomly assigned solution where their bodies were completely submersed. After 6 h each newt was removed from solution, patted dry, and weighed on the same balance. They were then returned to their solution and weighed again at 24 h. Percent body weight change from initial mass was calculated.

As body weight change is a classical metric in amphibian osmoregulatory physiology studies (e.g., Gordon et al. 1961), we placed our results for tidal *T. granulosa* in context with those from other well-known amphibian species inhabiting tidal and other saline habitats: *Fejervarya cancrivora* (Anura: Dicroglossidae) (Gordon et al. 1961), *Hoplobatrachus tigerinus* (Anura: Dicroglossidae) (Gordon et al. 1961), *Bufotes viridis* (Anura: Bufonidae) (Gordon 1962), and the salamander, *Batrachoseps gavilanensis*

(Caudata: Plethodontidae) (Licht 1975). We did not conduct any statistical comparisons of these species with our newt data, but rather present these for general information.

Laboratory Salt Challenge 2

a. Salt Stress and Blood and Tissue Collection

Male newts (33 from each population) were gradually acclimatized from 5˚C to 14˚C over thirteen days at the end of January 2013; they were then held at 14˚C for an additional three days before the start of experimentation. Newts were randomized to different lab-grade NaCl solutions (5.0 ppt, 10.0 ppt) or a freshwater control (0.2 ppt) (10-11 newts per treatment). Each newt was placed in 1.5 L of solution for 6 h. At 6 h, blood was sampled from the caudal vein (within 3 min) after snipping the end of the tail with a sterile surgical blade (French & Neuman-Lee 2012; Neuman-Lee et al. 2015). Blood was centrifuged at 2200 rpm to separate plasma from cells, and stored at -20˚C until used in osmolality, corticosterone and immune assays. All blood sampling was completed between 15:00-16:55. Tail snips were retained for Na^+/K^+-ATPase assaying and stored at -80˚C until assayed.

A total of three newts exposed to Control, two newts in 5 ppt, and six newts in 10 ppt in this experiment were previously exposed to salt in Laboratory Salt Challenge 1, three months earlier. There were no significant differences in responses of newts that had previously been exposed to salt in the first experiment versus those that were only exposed to salt in the second experiment (t-tests for all treatments, $p>0.05$), and all newts were housed in fresh water between experiments.

b. Plasma Osmolality

Osmolality of plasma samples were determined in triplicate using a vapor pressure osmomoter (± 6 mOsm; model 5600; Wescor Inc., Logan, Utah, USA), as described by Davis and DeNardo (2007). Two individuals displayed much greater than average standard error in their replicate readings and were not included in the final analysis. When analyzed, it became apparent that one individual freshwater animal in 10 ppt exhibited an abnormally low osmolality value (204.25 mOsm; mean ± SE osmolality for this group = 337.94 ± 7.17 mOsm), and as a result was highly skewing normality of the data. Removal of this outlier greatly improved normality of the distribution of data, and we have chosen to present the results of analyses with and without this outlier. Analyses were therefore completed on samples from a total of 11 freshwater and 10 tidal newts in control, eight freshwater and nine tidal newts in 5 ppt, and seven (six, excluding the outlier) freshwater and eleven tidal newts in 10 ppt.

c. Na^+/K^+-ATPase extraction and assay

A 5 mm sterile biopsy punch was used to collect a uniform sized sample of skin and tissue from newt tail snips for Na^+/K^+-ATPase extraction. Extraction and assaying followed Petschenka, Offe and Dobler (2012). Briefly, samples were homogenized in 500 µl distilled water using a 1 mL all-glass grinder. Homogenates were sonicated in a chilled bath for 15 min to further break apart cell membranes, and then centrifuged at 10,000 x g for 10 min to precipitate debris. The supernatant was then ultracentrifuged (Beckman Coulter; Optima TLX; rotor: TLA-100.3) at 84,000 x g for 30 min at 4˚C to sediment the membranes containing enzymes. The supernatant was discarded and the microsomal

pellet was washed in distilled water, then resuspended in 300 µl distilled water by vortexing and sonication. After reconstitution, samples were stored at -20˚C until assayed. Samples were thawed in a sonicator and thoroughly vortexed prior to assaying. Assaying was based on methods detailed by Petschenka et al. (2013). In brief, Na^+/K^+-ATPase activity was determined by the quantification of inorganic phosphate released from ATP after incubation with the samples. Phosphate was stained (Taussky-Shorr) and absorbance measured using a BioRad xMark Microplate Spectrophotometer. Na^+/K^+-ATPase activity was calculated by regressing the final absorbance (nM) values of the phosphate standards against their corresponding known concentrations, and using the resulting linear equation to determine the concentration of phosphate (mM) produced in the assay.

c. Corticosterone Radioimmunoassay

Circulating corticosterone concentrations were determined following Neuman-Lee et al. (2015) using a radioimmunoassay protocol modified from French, Fokidis and Moore (2008). Samples were assayed in duplicate (antibody: MP Biomedicals, Lot #3R3PB-19E), and final concentrations adjusted using individual recoveries. Intra-assay variation was 6.9%. This assay has been used for *Taricha granulosa* previously (Neuman-Lee et al. 2015), but was additionally validated using pooled samples. We tested for parallelism using a serial dilution with the following points: 1:1, 1:2, 1:3, 1:5, 1:10, for interference by spiking samples with known quantities of corticosterone (40 pg/ml, 250 pg/ml, and 1000 pg/ml), and for non-specific binding.

d. Immune Assay

We performed a bactericidal assay to assess immune functioning in newts following salt exposure, using the protocol outlined in French and Neuman-Lee (2012). Briefly, we combined a 1:5 plasma dilution with CO_2-Independent media plus 4nM L-glutamine, and 10^4 colony producing units *Escheria coli* (EPowerTM Microorganisms #483-237-1, ATCC 8739, MicroBioLogics, St. Cloud, USA), and agar broth on a 96-well microplate. We incubated the plate for 12 hours and calculated absorbance using a BioRad xMark microplate reader. Samples were run in duplicate, with means being used for subsequent analyses. Bactericidal ability was calculated by dividing the absorbance for each sample by mean absorbance for the positive controls (containing only media and bacterial solution), and multiplying by 100. This provided the percent bacteria killed relative to the positive controls. Negative controls (containing media only) were also run to ensure that contamination was absent. One freshwater individual in 10 ppt displayed much greater than average variation in replicates and was removed from analysis.

Statistical Analyses

For Laboratory Salt Challenge 1, we compared percent body weight change with a four-way repeated measures ANOVA with the variables: Salt Treatment (fixed effect factor; 3 levels: Control, 5 ppt, 10 ppt NaCl), Population (fixed effect factor; 2 levels: Fresh, Tidal), Sex (fixed effect factor; 2 levels: Male, Female), Time (2 time points), and their interactions as independent variables. After determining that Sex did not play a significant explanatory role ($F_{1,47}$=0.056, p=0.814), we analyzed percent change in body

weight with a three-way repeated measures ANOVA with Salt Treatment, Population, and Time (and their interactions) as the explanatory variables.

For Laboratory Salt Challenge 2, we analyzed the overall effect of Salt Treatment (fixed effect factor; 3 levels: Control, 5 ppt, 10 ppt NaCl), Population (fixed effect factor; 2 levels: Fresh, Tidal), and their interaction on plasma osmolality, Na^+/K^+-ATPase, and bactericidal ability of newts using two-way ANOVAs. Bactericidal ability was square-root transformed to meet the assumptions of normality and homogeneity of variance. Corticosterone concentration was analyzed using a generalized linear model with a gamma distribution and log link function. Finally, to investigate if there was any correlation between osmoregulation and physiological stress, we conducted non-parametric Spearman correlations of corticosterone concentrations and (i) plasma osmolality and (ii) Na^+/K^+-ATPase for both populations combined, and separately for each population. All statistical analyses were completed in SAS® v. 9.3, and R v. 3.03, with significance $\alpha=0.05$.

RESULTS

Field Observations

We measured over 60 newts from each of the tidal and freshwater areas of Hunter Creek (Table 7.1 Supporting Information; Fig. 7.1ab). There were no significant differences between fresh and tidal population morphometrics (Table 7.1 Supporting Information; all t-tests, $p>0.10$). Newts were observed foraging, seeking refuge, mating, and ovipositing at both locations, including mating as close as ~100 m from the ocean

(Fig. 7.1a,b). All adult males were in breeding condition. Most females were gravid and some were observed depositing eggs in vegetation, sand, and gravel in both tidal and freshwater sites (ZMH and GRH, pers. obs.). In the tidal area, newts were observed at high and low tide, in areas with plentiful littoral and shoreline vegetation, as well as areas devoid of vegetation, nearer the mouth of the stream. Eels (Anguilliformes), sculpins (Scorpaeniformes), and a dead sea star (Forcipulatida, *Pisaster* spp.) were also observed in this habitat (ZMH and GRH, pers. obs.). We (GRH and ZMH) returned to this creek in May 2013 and 2014 and again observed over 100 newts breeding in the tidal habitat. Thus, there appears to be a well-established, healthy breeding population of these animals inhabiting this environment.

Laboratory Salt Challenge 1: Weight Change Experiment

Newts from both populations maintained their mass over time in control solutions (Fig. 7.2). In contrast, salt treatment caused a significant increase in the mass of newts from both populations over time, with the greatest increases occurring in 5 ppt for freshwater animals (Fig. 7.2). There was a significant effect of salt treatment ($F_{2,53}$=30.93, $p<0.0001$), population ($F_{1,53}$=9.13, $p<0.01$), and their interaction ($F_{2,53}$=5.18, $p<0.01$), on percent body weight change. There was no significant effect of time ($F_{1,53}$=0.50, $p=0.483$), but there were significant interactions between time and population ($F_{1,53}$=5.24, $p<0.05$), and time and salt treatment ($F_{2,53}$=5.37, $p<0.01$). There was not a significant three-way interaction between time, salt treatment, and population ($F_{2,53}$=2.59, $p=0.084$).

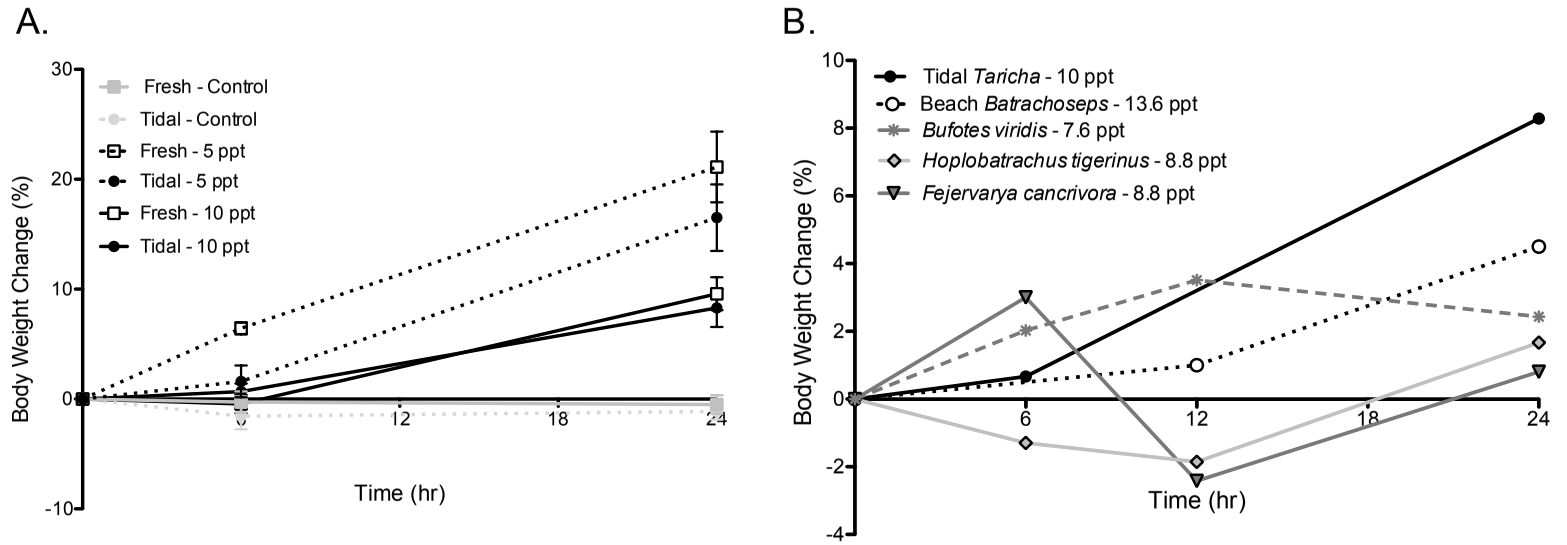

Fig. 7.2. (a) Mean (±SE) percent body weight change in rough-skinned newts (*Taricha granulosa*) from tidal (closed circles) and fresh (open squares) populations at Hunter Creek after immersion in control (0.2 ppt) (grey lines), 5 ppt (dotted black lines) or 10 ppt (solid black lines) NaCl solutions for 6 hours in Laboratory Salt Challenge 1. (b) Comparison of mean percent body weight changes of tidal *Taricha granulosa* in 10 ppt with other euryhaline amphibians in similar salinities taken at comparable times. Mean values for *Batrachoseps gavilanensis* found under driftwood on a beach (open circles, dotted black line) from Licht (1975). Values for *Bufotes viridis* (stars, gray dashed line) from Gordon (1962), and *Hoplobatrachus tigerinus* (diamonds, solid gray line) and *Fejervarya cancrivora* (triangles, solid dark gray line) found in mangrove swamps from Gordon et al. (1961). As the majority of these studies did not present any measure of variation (i.e., SE or SD), we present means here only for all data.

Laboratory Salt Challenge 2

a. Plasma osmolality

Plasma osmolality significantly increased with salt concentration, with newts having the highest plasma osmolality in 10 ppt (Fig. 7.3a). Including the outlier in the analysis (see Methods), yielded a significant effect of salt treatment ($F_{2,50}$=66.42, $p<0.0001$), though not of population ($F_{1,50}$=2.55, p=0.116) or the interaction term ($F_{2,50}$=0.392, p=0.678). With the exclusion of the outlier, salt treatment remained significant ($F_{2,49}$=115.67, $p<0.0001$), and population became a significant factor influencing plasma osmolality ($F_{1,49}$=8.27, $p<0.01$). However, the interaction between treatment and population remained insignificant ($F_{2,49}$=1.14, p=0.329). On average, tidal newts exhibited lower plasma osmolality than freshwater newts (Fig. 7.3a).

b. Na^+/K^+-ATPase

There were no significant effects of salt treatment ($F_{2,57}$=1.23, p=0.300), population ($F_{1,57}$=1.93, p=0.170), nor their interaction ($F_{2,57}$=0.022, p=0.978) on Na^+/K^+-ATPase production. Although not significant, there appears to be an overall trend of tidal newts having lower Na^+/K^+ pump activity than freshwater newts, regardless of salt treatment, and a slight increase in pump activity at 10 ppt vs. the other treatments (Fig. 7.3b).

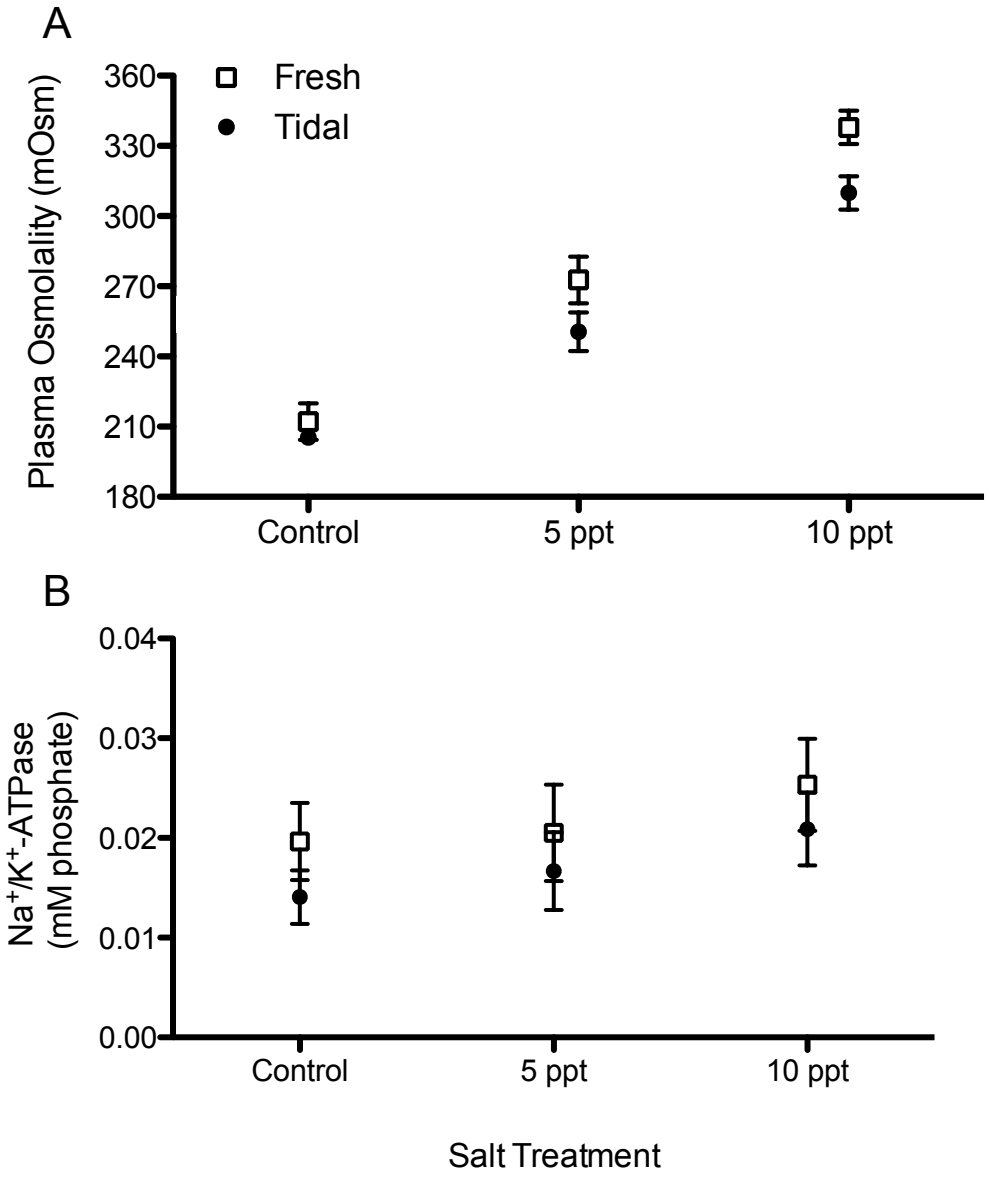

Fig. 7.3. Mean (±SE) (a) plasma osmolality (mOsm) and (b) Na^+/K^+-ATPase (mM phosphate produced) of tidal (closed circles) and fresh (open squares) newts after immersion in control (0.2 ppt), 5 ppt, or 10 ppt NaCl solutions in Laboratory Salt Challenge 2.

c. Corticosterone

There was an overall significant effect of salt treatment ($F_{2,56}$=5.01, p<0.01) and population ($F_{1,56}$=7.47, p<0.01), but not their interaction ($F_{2,56}$=2.24, p=0.116), on corticosterone levels of newts exposed to salt in the laboratory. Corticosterone levels increased 44% at 5 ppt and 63% at 10 ppt in freshwater animals compared to the control, but did not increase at 5 ppt, and only increased 36% at 10 ppt in tidal animals (Fig. 7.4).

There was a significant positive correlation between corticosterone and plasma osmolality (both populations combined: S=9561.23, p=0.00017, ρ (rho)=0.512; fresh only: S=772, p=0.0071, ρ=0.564; tidal only: S=1834.34, p=0.022, ρ=0.440), but not Na^+/K^+-ATPase (both populations combined: S=18286.2, p=0.647, ρ=0.067; fresh only: S=1356.4, p=0.294, ρ =0.234; tidal only: S=3439.12, p=0.805, ρ=-0.049).

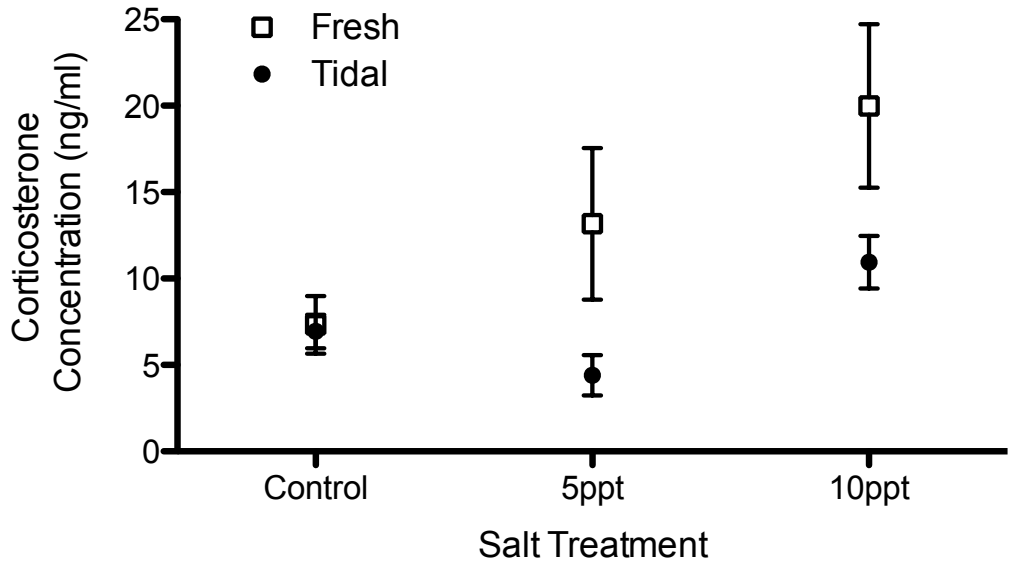

Fig. 7.4. Mean (±SE) blood plasma corticosterone concentration (ng/ml) of newts from tidal (closed circles) and fresh (open squares) populations after immersion in control (0.2 ppt), 5 ppt, or 10 ppt NaCl solutions in Laboratory Salt Challenge 2.

d. Immune Functioning

Newt immune functioning was significantly affected by salt treatment ($F_{2,59}$ = 5.20, $p<0.01$), but not population ($F_{1,59}$=0.899, p=0.347) or the interaction between treatment and population ($F_{2,59}$=0.754, p=0.475). Salt treatment, especially 5 ppt, appears to have an overall negative effect on newt immune functioning, regardless of population (Fig. 7.5).

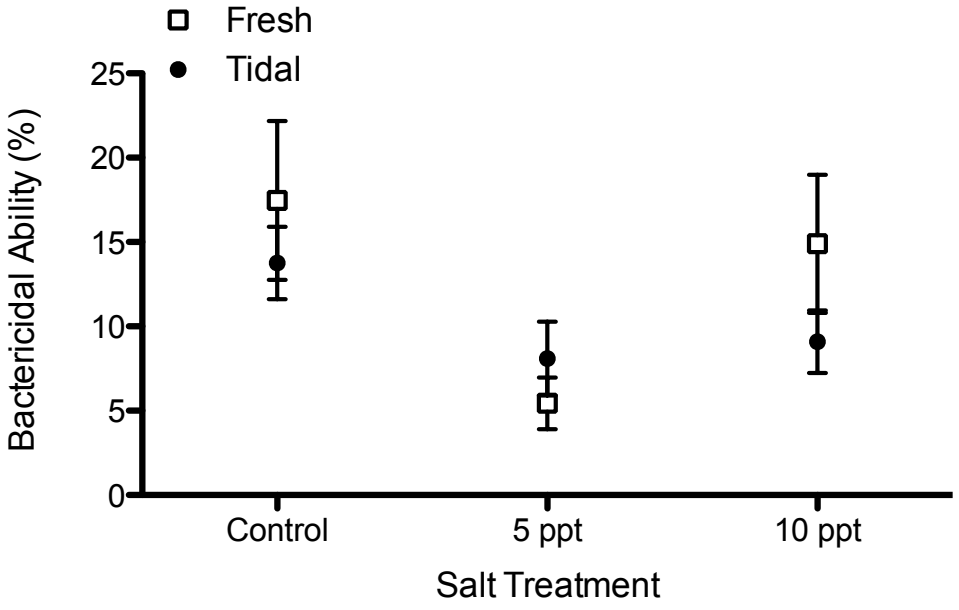

Fig. 7.5. Mean (±SE) percent of bacteria killed by blood plasma immune components in fresh (open squares) or tidal (closed circles) newts immersed in control (0.2 ppt), 5 ppt, or 10 ppt NaCl solutions in Laboratory Salt Challenge 2.

DISCUSSION

Exposure to salt water significantly affected the osmotic, stress, and immune physiology of rough-skinned newts (*Taricha granulosa*) from a coastal stream in Oregon. Salt water caused newts to disrupt their osmotic homeostasis (gain weight and increase plasma osmolality), increase plasma corticosterone concentrations, and decrease immune function (at 5 ppt), all indicators of physiological stress. In support of our hypotheses, we found population-level differences in responses to salt from animals living in different areas of the stream; newts from the tidal area showed reduced physiological responses to salinity as compared to newts from the freshwater area.

Salt exposure disrupted the physiological homeostasis of freshwater newts, as seen in their increase in body mass relative to control. Tidal animals, in contrast, did not show a large disruption in either solution (only small gains or losses occurred, relative to the freshwater animals in salt) (Fig. 7.2a). Disruption of osmotic homeostasis in response to salt is well-known and expected in amphibians, due to their highly permeable skin (Shoemaker & Nagy 1977). Similar patterns of initial increases in mass when placed in salt, possibly due to drinking or the temporary internal accumulation of urea, have been found in a number of other amphibian species (Fig. 7.2b). However, smaller increases seem to occur in saline-adapted species (i.e., *Fejervarya cancrivora* vs. *F. tigerina* (Gordon et al. 1961)) and populations (i.e., beach vs. inland populations of the salamander *Batrachoseps gavilanensis* (Licht 1975)), similar to our results with tidal and freshwater newts.

The internal osmolality of newts increased with environmental salinity in Experiment 2 (Fig. 7.3a). In response to external salinity changes, amphibians will often increase uptake of salts via integumentary sodium channels and pumps, as well as the hypersynthesis and retention of urea, to maintain an internal osmolarity iso-osmotic with the external environment (reviewed by Shoemaker et al. 1992; Katz 2015). It appears that while tidal newts were able to achieve this on average at 5 ppt (5 ppt = 256 mOsm external osmolality; mean plasma osmolality of tidal newts at 5 ppt = 250.55 mOsm), freshwater newts somewhat overshot iso-osmolality, with a mean plasma osmolality of 272.71 mOsm, making their body fluids slightly hyperosmotic to the surrounding environment. If water were to then follow this gradient via osmosis, this might explain why freshwater newts experienced a larger increase in body mass at 5 ppt than tidal newts (Fig. 7.1a). Beach-dwelling salamanders (*Batrachoseps gavilanensis*) that gained less weight in salt water than their inland counterparts also maintained lower internal salt concentrations than inland animals when exposed to salt stress (Licht 1975); this was primarily due to lowered plasma Na^+ concentrations (Licht 1975). While we do not know the chemical makeup of the plasma of *Taricha granulosa* in our study, Brown, Savage and Brown (1988) found that newts exposed to salt-depleted water decreased their plasma osmolarity primarily due to decreased Na^+ concentrations, which was correlated with a decrease in skin electrical activity (potentially due to decreased Na^+ pump activity). In our study, there was a general trend of increasing Na^+ pump activity at 10ppt, with tidal newts having an overall lower Na^+/K^+ pump activity compared to freshwater newts regardless of treatment (Fig. 7.3b). Thus, control over Na^+ uptake through the skin may be an osmoregulatory mechanism used by this species. The trends

in Na^+ pump activity, however, were not statistically significant. It is likely that this result is due to our choice in assaying skin from the tail. It may be that osmotic exchange is more active in other areas of the body, such as the ventral torso (Shoemaker & Nagy 1977; Toledo & Jared 1993). In addition, although many amphibian species tend to primarily use Na^+ ion regulation to osmoregulate (Katz 2015), other species primarily osmoregulate through retaining and oversynthesizing urea (Katz 2015). Although Licht (1975) did not implicate urea as important for the osmoregulation of *Batrachoseps gavilanensis*, Jones and Hillman (1978) found that it was important in a related *Batrachoseps* species, and it may be that *T. granulosa* also use urea to osmoregulate. Future studies should examine concentrations of specific ions and urea in the plasma to elucidate a more precise osmoregulatory mechanism. In contrast to 5 ppt, at 10 ppt neither freshwater nor tidal newts appeared capable of achieving iso-osmolality with the external environment (10 ppt = 512.8 mOsm; highest plasma osmolality of any newt at 10 ppt = 360 mOsm). This fact may help explain the otherwise unexpected result of lower body weight change at 10 ppt than 5 ppt as seen in Experiment 1. If the internal osmolality of newts is lower than the external environment, water will not enter the newts' body through osmosis, and therefore not cause an increase in weight. While less so than freshwater newts, tidal newts do increase their plasma osmolality significantly (and trend towards increasing Na^+ pump activity) in 10 ppt compared to other treatments.

The osmoregulatory realities for newts from the two populations may be evident in other physiological metrics, such as the stress response. Indeed, the dose-dependent relationship between salinity and corticosterone (Fig. 7.4) seen in freshwater newts is indicative of physiological stress. There was a significant positive correlation between

corticosterone and plasma osmolality, suggesting that larger internal changes are indeed more stressful. Increases in corticosterone with salinity have also been seen in larval salamanders (*Ambystoma jeffersonianum*) (Chambers 2011). This relationship is not as strong in tidal newts, however, and in fact appears to only manifest at 10 ppt, when osmoregulation becomes more challenging. It may be that tidal newts do not live in an environment that typically reaches this salinity, or it may simply be that this represents a more general limit to amphibian salt tolerance, even for saline-adapted species (Gomez-Mestre & Tejedo 2003; Hopkins & Brodie 2015). Below 10 ppt, concurrent with the apparent trends seen in osmoregulatory physiology, it appears that tidal newts do not respond to increased salinity as stressful, and thus their threshold for initiating a physiological stress response may be higher than their conspecifics that do not live in this environment (Romero, Dickens & Cyr 2009).

Our results also indicate that salt-induced physiological stress has the potential to have downstream effects on immune functioning (Fig. 7.5), even with only acute exposure. However these effects are not as clear-cut as those on osmoregulation and stress physiology, and vary according to concentration and population. Bactericidal ability of plasma from freshwater newts was only reduced at 5 ppt, but not at 10 ppt. In contrast, tidal newts showed a non-significant decrease at 5 ppt and 10 ppt. One likely explanation for our non-linear immune results is that the effects of corticosterone are context dependent, where they are suppressing the immune system in some instances (e.g. chronic duration) and enhancing it in others (i.e., acute duration) (Dhabhar 2009). The increase in corticosterone in both populations at 10 ppt (though larger in freshwater newts) (Fig. 7.3) may offset negative immune effects due to salt stress found at 5 ppt.

Further research should thus focus on the nuanced effects of different types of stress to partition out the true effects of salt stress itself versus corticosterone on the immune system. Regardless, salt stress does seem to alter immunity, which is critical for individual health and lifetime fitness (Lochmiller & Deerenberg 2000; Ricklefs & Wikelski 2002; Rolff 2002), and is crucial when considering fitness effects on populations.

While we found clear population differentiation in the osmotic and stress physiological responses of tidal vs. freshwater newts to salinity, the mechanisms behind such differentiation remain less clear. Population differentiation may be the result of local adaptation, maternal effects, phenotypic plasticity, or even developmental priming early in life. We did take steps to minimize phenotypic plasticity by housing the two populations in a common garden environment in the lab for several months before physiological testing. However, as only adult newts were collected and tested in this study, it is impossible to rule out the effects of early life exposure to potentially increased salinity in the tidal area affecting the physiological plasticity of tidal individuals later in life (although exposure to transient salinity early in life has recently been shown to have no effect on salinity tolerance in later life in another amphibian (Kearney et al. 2014)). In addition, while population differentiation (or at least a trend thereof) was evident in many physiological responses, it would be prudent to point out that there was also high individual variation in responses from newts from both populations. This may be a product of the small sample sizes used in our laboratory experiments, the relatively brief nature of the experiments, or potentially a very real phenomenon characterizing these populations in this environment. A previous study found high intrapopulation variation

in salinity tolerance in an inland, salt-naïve newt population (Hopkins et al. 2013), and there may likewise exist high intra-population variation in salt tolerance in these coastal populations. In addition, this stream is continuous habitat for newts, and while the "populations" in our study are separated by over 3km, there is also likely migration and gene flow between the populations, which may act to dampen patterns of population differentiation (Lenormand 2002). We currently know very little about the genetic nature of salinity tolerance in amphibians (Hopkins & Brodie 2015), and more work is needed to disentangle the genetic or plastic nature of how tidal newts survive in this stressful environment.

Regardless of its ultimate cause, the fact that tidal newts do not appear to be as physiologically stressed by salt compared to conspecifics upstream suggests that this population may have some resilience to intermittently increased salinity in this habitat. Gunzburger et al. (2010) also found amphibian communities living in normally freshwater coastal wetlands on the east coast of the United States were remarkably resilient to salinity increases due to hurricane storm surges, and Moreira et al. (2015) found a similar result with Brazilian tadpoles subjected to unpredictable intermittent salinity due to artificial estuary breaching. Amphibians are found in coastal environments around the world, often in habitats affected by storms and sea-spray (Hopkins & Brodie 2015). As the rate of storm events, coupled with sea-level rise increases in these habitats, physiological strategies for coping with intermittent salinity may be increasingly necessary for many species. More broadly, transient osmotically stressful events are found in both coastal and non-coastal environments, including habitats impacted by road deicing salts. After road deicing events, salinity in normally

fresh road-side aquatic habitats spikes dramatically for a brief period (Whitfield & Wade 1992; Whitfield & Wade 1996), not unlike a coastal storm event (Fig. 7.1c). Thus, amphibians and other osmotically sensitive freshwater organisms have to cope with unpredictable salinity in both coastal and inland environments, and possibly adapt (e.g., Brady 2012) in order to survive in these stressful habitats.

As environments become more unpredictable, understanding the physiological responses of animals and their ability to survive in the presence of changes will only become more critical to the conservation of biodiversity (Hopkins & DuRant 2011). Our ability to place physiology in the context of these environments, events (Wingfield & Kitaysky 2002; Wingfield 2013), and the larger adaptive landscape (Parsons 2005), will be key to gaining these insights.

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SUPPORTING INFORMATION

Table 7.1. Numbers and morphometrics of wild-caught rough-skinned newts (*Taricha granulosa*) from the tidal and freshwater areas of Hunter Creek, Oregon (SVL=Snout-vent Length, TL=Total Length). There are no significant differences between populations of same sex in any metric (all t-tests, $p>0.10$).

Population	Sex	N	SVL (cm)		TL (cm)		Mass (g)	
			Mean	SE	Mean	SE	Mean	SE
Fresh	Male	33	8.05	0.090	18.04	0.232	18.99	0.556
	Female	30	7.02	0.075	14.25	0.151	12.93	0.306
Tidal	Male	37	7.91	0.075	18.2	0.202	18.48	0.448
	Female	29	6.85	0.105	14.37	0.179	12.13	0.380

Salinity and temperature environmental measurements:

We took temperature and salinity measurements of the water in Hunter Creek on multiple days and times, at both high and low tides, using a hand-held YSI EC300® multimeter (YSI Inc., Yellow Springs, USA). Water throughout the tidal area was fresh overall, with salinity 0.1-0.5 ppt at both high and low tides. We did find elevated salinity (1.4 ppt [a concentration detrimental to newt embryonic and larval survival and development (Hopkins, French & Brodie 2013b; Hopkins, French & Brodie 2013a; Hopkins, Brodie & French 2014)]) in certain shallow areas of the stream with newts at low tide, but salinity varied from day to day and hour to hour. Taking salinity measurements at any one point in time should be treated as a "snapshot" only, and not necessarily the potential range that the habitat or its inhabitants may experience. For example, dramatic storm events also wash seawater upstream throughout the tidal area

from time to time (Fig. 1c) (however, we were unfortunately unable to measure salinity during such an event). The freshwater area of the stream was consistently fresh, 0.0-0.1 ppt.

References for Supporting Information:

Hopkins, G.R., Brodie, J., E.D. & French, S.S. (2014) Developmental and evolutionary history affect survival in stressful environments. *PLoS One,* **9,** e95174.

Hopkins, G.R., French, S.S. & Brodie, J., E.D. (2013a) Increased frequency and severity of developmental deformities in rough-skinned newt (*Taricha granulosa*) embryos exposed to road deicing salts (NaCl & MgCl2). *Environmental Pollution,* **173,** 264-269.

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CHAPTER 8

SUMMARY & CONCLUSIONS: AN EVOLUTIONARY PERSPECTIVE

SUMMARY

Despite amphibians being osmotically challenged organisms, salt tolerance has evolved a surprising number of times in numerous families of frogs, toads, salamanders, newts, and (maybe) even caecilians all around the world (Ch. 2). This dissertation examined the evolutionary potential for rough-skinned newts (*Taricha granulosa*) to join this group of animals.

We found that substantial interfamily variation exists in a variety of newt early life-history traits important for fitness, and that this variation persisted regardless of maternal or environmental (temperature) effects (Ch. 3). This variation provides the raw material for natural selection to act on newt early life-history traits, and is thus an important prerequisite for evolution.

We confirmed that salt is a powerful agent of selection in these amphibians, severely affecting embryonic (Ch. 4) and larval (Ch. 6) survival, embryonic development, growth, time to hatching (Ch. 4) and the frequency and severity of developmental deformities (Ch. 5). In addition, sub-lethal effects at the embryonic stage, especially in terms of embryonic growth and size at hatching, can have critical carry-over effects on larval survival post-hatching (Ch. 6). Importantly, we also ran some of the first tests of the effects of the emerging deicing salt, $MgCl_2$ on amphibian embryonic survival and development, and discovered that it is just as potent as the more traditionally used NaCl

(Ch. 4, 5). Larval newts are even more greatly affected by $MgCl_2$, and we hypothesize that this is related to a lack of evolutionary history newts have with physiologically regulating $MgCl_2$ in their habitats, in contrast to the more commonly found NaCl (Ch. 6). Such detailed quantification of all these effects, at and across life-history stages, is a hallmark of this dissertation research, and provides important details as to the actual severity and selective pressures that salts provide.

While the destructive effects of salt on amphibians was confirmed in this research, and we have provided much needed new detailed data on effects of specific road deicing salts, perhaps the most critical knowledge gained from this dissertation research is that amphibians may not be helpless victims to salt either, and that the evolutionary potential exists for salt naïve rough-skinned newt populations to adapt through natural selection exploiting standing variation in salinity tolerance (Ch. 4). That such interfamily variation in critical early life-history traits exists in a newt population (Ch. 3), and that salt can act on that variation (Ch. 4) is key to understanding the ability of this species to adapt to both anthropogenic and natural sources of salt (however newts may be more limited in their ability to adapt to $MgCl_2$ vs. NaCl - Ch. 6).

The ability of newts to exploit this evolutionary potential and adapt to natural sources of NaCl in their environment was investigated through the discovery of a population of newts inhabiting the tidal area of a coastal estuarine stream, ~100 m from the Pacific ocean (Ch. 7). By comparing physiological responses of these animals to newts living 3 km upstream, we found that tidal newts appear to be physiologically adapted to salt water, with salinity causing fewer disruptions from osmotic, stress, and immune homeostasis compared to freshwater newts found further upstream (Ch. 7). The

unpredictable nature of salinity events in the tidal area of this stream is a tangible demonstration of the transient nature of salinity in freshwater habitats around the world, impacted by both natural and anthropogenic sources of salt, and inhabited by newts and countless other amphibians (Ch. 2).

We conclude this dissertation by discussing an evolutionary perspective on salinity adaptation in amphibians in general, areas where further research is needed, and broad lessons learned.

TOWARD AN EVOLUTIONARY MODEL OF AMPHIBIAN ADAPTATION TO SALINE HABITATS[7]

Genetic Nature of Salinity Tolerance

Populations can adapt to novel or challenging environments in two ways, either through the propagation of new mutations or through natural selection acting on standing genetic variation in traits (Barrett and Schluter 2007). While the propagation of new mutations can be effective, it is generally a much slower process with a lower probability of fixation than selection exploiting existing standing genetic variation (Barrett and Schluter 2007). Surprisingly few studies have examined standing variation for salinity tolerance in amphibians, but those that have looked (Roberts 1970; Gomez-Mestre and Tejedo 2003,2004; Hopkins et al. 2013b) have found it. Significant variation in salinity

[7] This section of Chapter 8 has been modified slightly from a paper coauthored by Gareth R. Hopkins and Edmund D. Brodie, Jr. Reprinted with permission from Allen Press from Herpetological Monographs Vol. 29, pages 1-27, 2015.

tolerance has been examined among sibships of Natterjack Toads (*Epidalea calamita*) inhabiting fresh and saline lakes in Spain (e.g., Fig. 2.5D) (Gomez-Mestre and Tejedo 2003,2004), as well as in Pacific Tree-Frogs (*Pseudacris regilla*) in Oregon, USA (Roberts 1970). Roberts (1970, p. 32) wrote: "It was observed that, even in tests run on animals from salt sensitive areas, there were always a few animals that survived the highest levels of salt in the test solutions. This suggested that there was at least a measure of plasticity, with respect to salinity tolerance, in the gene pool." These findings are similar to what has been found with Rough-Skinned Newts (*Taricha granulosa*) (e.g., Fig. 2.5C) on the Pacific Coast of North America (Hopkins et al. 2013b). In a salt-naïve population inhabiting an inland pond, some females had 100% survival of eggs in salt water, whereas others from the same population had 100% mortality, representing a significant female x salt interaction, and the potential for local adaptation (Hopkins et al. 2013b). In a coastal stream, there was also variation in physiological responses of adult newts to salinity stress, although we do not know the exact nature of this variation (Ch. 7).

While variation is critical for natural selection, it is so only in the degree to which it is heritable. Broad-sense heritability in salinity tolerance has been found in locally-adapted Natterjack Toad (*Epidalea calamita*) populations, which increased with increased salinity (up to $H^2 = 0.50$) (Gomez-Mestre and Tejedo 2004). However, narrow-sense estimates of heritability did not necessarily follow the same pattern, possibly due to other additive effects, including maternal effects. Maternal effects of female or egg size were not found, however, to have any significant effect on either local adaptation (Brady 2012), or variation (Hopkins et al. 2013b) in other salt-tolerant amphibians (tests on

eggs). Thus, there appears to be modest evidence that salt tolerance is genetic in nature. The fact that a high degree of genetic population subdivision (high inter-population Q_{ST} values) for salinity tolerance persists in *E. calamita* is especially important in the face of molecular evidence indicating otherwise little genetic population differentiation and moderate to high gene flow (F_{ST}) between fresh and saline populations of toads (Gomez-Mestre and Tejedo 2004). We also found significant population differentiation in salinity tolerance between fresh and tidal rough-skinned newt (*Taricha granulosa*) populations within the same stream, which were presumably also connected by migration and gene flow (Ch. 7). The fact that local adaptation may have occurred in populations of toads (and possibly newts) in the face of this significant gene flow and presumed migration reveals the intense nature of the selective pressures of salinity.

To date, these studies remain the only hints of the genetic nature of salinity adaptation in amphibians. To the best of our knowledge, no molecular studies have identified genes for salt tolerance in amphibians or compared the genetic profiles of locally adapted populations. While very little knowledge is currently available on genetic variation in salt tolerance within populations, we predict that, if examined, one would find significant standing genetic variation in salinity tolerance in salt-naïve populations of many amphibian species, and that this variation is key to their adaptive ability. The sheer number of salt-tolerant species worldwide, and their apparently deep evolutionary relationships (Fig. 2.1) indicates that alleles for salinity tolerance (if they exist) in amphibians are most likely old and have been "pre-tested" by selection (Barrett and Schluter 2007) in many species and populations inhabiting naturally-saline environments. This would help explain the rapid evolution of salt tolerance observed in some inland

populations in response to anthropogenic application of salt (e.g., Brady 2012; Fig 2.3C), as the pace of evolution by natural selection is much faster with standing genetic variation than for new mutations. Adaptation to anthropogenic change has indeed been predicted to be primarily the result of standing genetic variation (Barrett and Schluter 2007; Bell 2013). Fully understanding the genetic nature of salinity tolerance clearly is the biggest, and most pressing, hurdle that remains in our elucidation of the evolution of amphibian adaptation to both natural and anthropogenic salt.

Origins of Salt Tolerance

The fact that alleles for salt tolerance might exist in amphibian populations is not surprising considering the primary importance of osmoregulatory functioning in these animals. Amphibians, due to their permeable skin and egg membranes, are highly sensitive to water loss at all life-stages and in all its forms (Shoemaker and Nagy 1977; Katz 1989). At the same time, amphibians generally live in environments deficient of salts, and thus their skin has evolved to be very efficient at transporting Na^+ and Cl^- ions into the body (Shoemaker and Nagy 1977). Efficient osmoregulation is a key trait under intense selective pressure in these animals. This is true whether the animal lives in arid conditions, where it must burrow in soil to aestivate, or in hyperosmotic saline aquatic systems, and amphibians can be found in both habitats (Katz 1989). Indeed, some species found in both arid and saline aquatic environments, such as the toad *Bufotes viridis* (= *balearicus*, Fig. 2.5A) (Degani et al. 1984; Katz 1989) and the salamander *Ambystoma tigrinum* (Delson and Whitford 1973), appear to use the same osmoregulatory physiological mechanism, overactive urea synthesis and retention, to

achieve tolerance of hyperosmotic conditions in both habitats. Thus, salinity tolerance in amphibians might have its evolutionary origins as an exaptation of tolerance to arid conditions (Gomez-Mestre and Tejedo 2005). Conversely, adaptation to arid conditions might be an exaptation to salt tolerance (Hoffman 2014). It has been proposed, for example, that *Bufotes viridis* initially evolved in aquatic environments with fluctuating salinity, and then dispersed to arid environments once this adaptation to increased ion concentrations had evolved (Hoffman 2014). Degani (1981) found support for a link between aridity and salinity tolerance in *Salamandra salamandra*, as salamanders from semi-arid areas of Israel were more tolerant of saline aquatic conditions than animals from moist habitats. When explicitly testing this exaptation hypothesis with the toad *Epidalea calamita*, however, Gomez-Mestre and Tejedo (2005) could find no support for it, and concluded that drought tolerance and salinity tolerance may have evolved independently in this species. Support for this conclusion also comes from the fact that the osmoregulatory physiological mechanisms amphibians employ pre- and post-metamorphosis appear to be fundamentally different, with larvae regulating salts through ionic exchange, and juveniles and adults primarily (but not exclusively - see Ch. 7) relying on the overactive synthesis and retention of urea (Gomez-Mestre et al. 2004; Gomez-Mestre and Tejedo 2005; Bernabò et al. 2013). As the physiological mechanism larvae employ for regulating increased ion concentrations would not work for post-metamorphic individuals facing drought conditions, this decoupling of osmoregulatory mechanisms pre- and post-metamorphosis suggests that drought tolerance and salinity tolerance may have evolved independently (Gomez-Mestre and Tejedo 2005). In addition, although there are certainly amphibian species such as these that occur in both

arid and saline habitats, there arguably are more that occur in coastal habitats (Fig. 2), where salinity tolerance in the face of oceanic salt would be highly beneficial. In direct contrast to Darwin's (1859) views on the matter, it now appears that salt tolerance in coastal amphibians may have resulted in the ability of these animals to disperse across oceans (Vences et al. 2003; Measey et al. 2007).

It is possible that ecological factors including biotic interactions could drive diversification of amphibians into saline habitats. Salinity is known as a driving force governing the composition of aquatic ecological communities (Gunter 1956), and recent work has suggested that salinity can affect the interactions of amphibians with other community members (e.g., food-web dynamics) both directly and indirectly (Petranka and Doyle 2010; Chambers 2011; Van Meter et al. 2011; Petranka and Francis 2013; Moreira et al. 2014; Van Meter and Swan 2014). Adaptation to salinity could certainly lead to novel predation opportunities for amphibians in these environments, as has been shown in some South American anurans eating marine invertebrates, for example (Sazima 1971; Brasileiro et al. 2010; Ferreira and Tonini 2010). It is also possible that salinity intolerance of freshwater invertebrate predators could lead amphibians to adapt to saline habitats to escape predation pressure (Moreira et al. 2014) (although this must be balanced by potentially increased pressure from marine predators (Pyke et al. 2013)). Differential susceptibility to salt can also affect amphibian species diversity and community composition in saline habitats (Karraker et al. 2008; Collins and Russell 2009; Karraker et al. 2010; Brown and Walls 2013; Gallagher et al. 2014; Moreira et al. 2014). Thus, an escape from competitors or predators, or novel prey opportunities (in short, changes in community composition and structure) may be driving forces in the

evolution of salt tolerance in amphibians. Research on this topic, however, remains relatively speculative and correlative at this time. The demonstration of definitive causal links between salinity, community composition, ecological interactions, and selective advantages for amphibians still needs to be completed and is an important endeavor for future investigation.

The Nature of Selection in Osmotically Stressful Environments

For amphibians in osmotically stressful environments, events that favor salinity tolerance may be predictable or unpredictable, which can have important consequences for evolution (Badyaev 2005; Parsons 2005). Regular, predictable exposure to salt is typified by amphibians inhabiting mangrove swamps, where daily tidal cycles temporarily increase salinity in a predictable way (e.g., Jena et al. 2013). The most familiar example of this is the Crab-eating Frog of Southeast Asia, *Fejervarya cancrivora* (Fig. 5B), the most well known euryhaline amphibian, whose physiological mechanisms for dealing with this predictable source of salinity were described by Gordon et al. (1961). In addition, amphibians may be able to adapt with the help of gradual acclimation to gradually increasing salinity in some environments where salinity is primarily elevated through evaporation (Gomez-Mestre and Tejedo 2003; Wu et al. 2014). Although these selection pressures may be common in some environments, amphibians in many other environments may experience much more unpredictable, dramatic salinity selection events. Indeed, it has been argued that dramatically fluctuating salinity levels are the norm, rather than the exception, in most environments (Wu et al. 2012; Kearney et al. 2014).

Stochastic coastal storm events can periodically wash seawater into otherwise mostly freshwater or tidal habitats (Thirion 2002; Gunzburger et al. 2010; Pyke et al. 2013; Hopkins and Hopkins in press; Ch. 7 - Fig. 7.1). This habitat can thus change dramatically and unpredictably, and so even though an area may be fresh for much of the time, extreme "pulses of selection" exist to maintain saline adapted animals in this habitat (Gunzburger et al. 2010). Bell (2013, p. 3) notes, "a catastrophic event that threatens the survival of a population is likely to occur only at long intervals, but when it does occur, it will have a decisive effect on the subsequent history of that population, because the resistant types that survive may have previously been very rare. Thus, the long-term fate of a population will often be governed by the extreme values of environmental and genetic variation." Most amphibians found in coastal habitats live in rock pools, streams, and beach areas affected by sea-spray, waves and storms (Table 2.1, Fig. 2.3A,D, Fig. 7.1). Roberts (1970) typified these observations for coastal Pacific Tree-Frogs (*P. regilla*): "One population sampled in this study came from a 'freshwater' pool within 5 meters of mean high tide and the tadpoles and eggs were collected in a shower of salt spray." Amphibians in coastal areas, including tidal newts (Ch. 7), increasingly have to deal with storm surges and inundations of habitats with seawater during extreme weather events (e.g., tsunamis, hurricanes, etc.) as they increase in frequency with climate change (Thirion 2002; Gunzburger et al. 2010; Brown and Walls 2013). Thus, amphibians in these habitats have been forced to evolve tolerance in response to these intermittent salinity events (Gunzburger et al. 2010; Brown and Walls 2013; Moreira et al. 2014; Ch. 7). The salinity of coastal areas can also be affected by anthropogenic management

activities, such as artificially opening and closing estuaries, resulting in the same pattern of disruptive, intermittent salinity inundation (Moreira et al. 2014).

Road deicing events also result in extreme transient spikes of salinity in roadside aquatic habitats, not unlike a coastal storm event (Whitfield and Wade 1992,1996), and habitat degradation and the changing of agricultural practices are also leading to extreme and unpredictable fluctuations of salinity in many inland habitats (Kearney et al. 2014). Unpredictable episodes of selection therefore probably play some of the most important roles in amphibian adaption to salt in both inland and coastal natural and anthropogenically-altered environments. Our understanding of how amphibians adapt to these fluctuating environments is still, however, mostly unexplored. Kearney et al. (2014) provide a much-needed first look at this subject, and their results suggest that animals experiencing transient salinity react very differently than those experiencing constant salinity. Our work with tidal newts (Ch. 7) demonstrates that relatively short-term (6 hours), transient exposure to salt can significantly disrupt physiological homeostasis of freshwater newts, but that it seems possible for tidal newts to adapt for the most part to this level of exposure. However, exposure for a longer, less transient, period of time (24 hours) disrupted the osmotic homeostasis (weight change) of otherwise adapted tidal newts. Much more work is needed on this subject, as understanding the frequency, predictability, and nature of selection events clearly is key to our understanding of adaptation in these environments (Parsons 2005; Bell 2013).

Limitations to Salt Tolerance

A final note should be made on possible limits to amphibian adaptation to salt. While there is extensive and important literature on the limitations of adaptation in general (Parsons 2005; Bell 2013), specific points salient to amphibian salt tolerance in particular can be made here. First, as discussed in Chapter 2, there may be a general upper limit of salt concentration (~10 ppt) that most amphibians living in saline habitats can tolerate. This limit was proposed by Gomez-Mestre and Tejedo (2003) over ten years ago, determined to be true in general for amphibians in our review (Hopkins and Brodie 2015; Ch. 2), and for newts in particular in Chapter 7. It was only when salinity reached 10 ppt that the physiological homeostasis of tidal newts was disrupted (Ch. 7). Despite this general upper limit, however, there are also clearly some amphibian species that can tolerate much higher salinities (up to 39 ppt; Fig. 2.7).

One of the ways that euryhaline amphibians such as *F. cancrivora* and *B. viridis* are able to tolerate such high salinities in the laboratory is through gradual acclimation to increasing salinity (Gordon et al. 1961; Gordon 1962; Gordon and Tucker 1968; Katz 1973). Acclimation may increase tolerance in these and other species (e.g., Licht 1975; Wu et al. 2014), through physiological means such as increased Na^+/K^+-ATPase expression, allowing larvae to more efficiently maintain osmotic homeostasis (Bernabò et al. 2013; Wu et al. 2014). The effects of acclimation do not appear to be universal, however, and in some cases may have either no effect (Kearney et al. 2014), or even inhibit adaptation (e.g., Hua and Pierce 2013). Acclimation to gradually increasing salinities may be a realistic ecological scenario in some habitats, such as saline desert ponds, where evaporation leads to increasing salinity over time (Gomez-Mestre and

Tejedo 2003), but may be less ecologically realistic in other habitats, where salinity may be governed more by dramatic unpredictable events, such as storms and road deicing salt application (see above; Hopkins et al. 2014 (Ch. 6); Ch. 7). Many species may therefore be limited in their adaptive abilities by a lower (but still effective and ecologically realistic) salinity limit to which they can respond immediately, without the need for gradual acclimation.

Despite the evidence that amphibian populations can locally adapt to saline environments, for some populations evidence is emerging that this may not always be possible (Brady 2013). In habitats subjected to anthropogenic salt, the pace of salinization may take place faster than adaptation can occur, and this, combined with severely reduced population sizes, a loss of genetic diversity, asymmetrical gene flow, altered migration, and inbreeding depression due to habitat fragmentation and degradation from multiple stressors, may limit the evolutionary responsiveness of these populations (Bell 2013; Brady 2013). These processes can result in maladapted vs. locally adapted populations, as has been found in Wood Frogs (*Lithobates sylvatica*) inhabiting anthropogenically salted roadside ponds (Brady 2013). These populations continue to persist, however; thus, while the animals may experience lower survival, increased malformations, etc. in their home roadside environment (Brady 2013), this also does not necessarily preclude them from inhabiting this habitat. Spotted Salamanders (*Ambystoma maculatum*) also appear to be able to locally adapt to increased salinity in these same ponds (Brady 2012), and Wood Frog populations have been found elsewhere inhabiting saline environments, such as tidal marshes (Ouellet et al. 2009). These

limitations to adaptation thus appear to be species-, population-, and habitat-specific, and more work is needed to be able to predict the responses of populations to salinity.

A final limit to adaptation, especially relevant in anthropogenically altered saline habitats, is the chemical nature of the salt and the evolutionary history amphibians have in regulating it. Several amphibian species, otherwise somewhat tolerant of NaCl, have been found to be susceptible to non-NaCl based salts, such as $MgCl_2$ (Dougherty and Smith 2006; Harless et al. 2011). In Rough-Skinned Newts (*T. granulosa*), significant interfamily variation exists in egg survival in response to both NaCl and $MgCl_2$, which affect eggs similarly (Ch. 4; Hopkins et al. 2013b). However, larval survival is significantly lower in $MgCl_2$ than in NaCl (Ch. 6; Hopkins et al. 2014), as has been found in anuran tadpoles (Dougherty and Smith 2006; Harless et al. 2011). It appears that eggs do not have substantial osmoregulatory ability and therefore are equally affected by both salts, whereas larvae have the ability to regulate Na^+, but not Mg^{2+}, through gill and integumentary Na^+ pumps (Ch. 6; Hopkins et al. 2014). This probably reflects the long evolutionary history that amphibians have with NaCl, but not $MgCl_2$, in various naturally saline habitats around the world (Drever 1997). Na^+, but not Mg^{2+}, also has a long evolutionary history as being one of the most common vertebrate osmolytes (Shoemaker and Nagy 1977). Thus, it appears that the adaptive ability of amphibians to particular types of salt may be limited by their physiological means of regulating the salt in question, a product of their evolutionary history with the chemical (Ch. 6; Hopkins et al. 2014). This has particularly important consequences for the ability of amphibians to adapt to anthropogenic sources of salt, such as road deicing salts, which are often increasingly not NaCl based (e.g., Harless et al. 2011). $MgCl_2$ is now the second most

commonly used road deicing salt in North America (National Transportation Research Board 2007) and is used exclusively in some regions. Amphibian populations in these areas may thus be constrained in their ability to adapt to this evolutionarily more "foreign" salt. Future management decisions regarding the selection and application of road deicing salts should take into account this evolutionary perspective (Hopkins et al. 2014).

LESSONS LEARNED FOR STUDYING EVOLUTION IN STRESSFUL ENVIRONMENTS

This dissertation has hopefully succeeded in exploring in depth the question of amphibian adaptation to saline environments. However, I believe that some of the most important lessons learned from this process have broad implications for understanding evolution in stressful environments across a wide range of study systems and stressors, and are worth briefly reviewing.

Variation is important - Variation is the raw material for natural selection, and where heritable, can lead to evolutionary change. This is the underlying basis for local adaptation, and is thus critical for examining evolutionary responses to environmental change, regardless of organism or stressor (Kawecki and Ebert 2004; Barrett and Schluter 2007; Carroll et al. 2007; Bell 2013). When examining the responses of individuals in a population to a stressor, it is clearly not enough to look only at mean responses, as has been done traditionally in the eco-toxicological, environmental science, and conservation

literature. The mean responses of newt embryos from all the females in the population we studied to road deicing salts indicate unsurprisingly that increasing salt leads to increasing mortality. It is only when the variation in responses is examined that we begin to understand the whole picture: this population has the potential to adapt through natural selection, and thus survive in this stressful environment (Ch. 4; Hopkins et al. 2013b). Organisms around the world are resilient and highly adaptable. Darwin (1859) realized that every population contains variation in key survival traits, and that this was the key for evolution. I would argue that it is the key for adaptation and the survival of many species in an increasingly stressful world, and should always be examined.

Developmental and evolutionary history matter - Stressors can both differentially affect different life-history stages, and have significant effects across stages. Understanding the developmental history of an organism with its stressor is key to fully understanding the selective pressures that the stressor causes, and the differential ability of different life-history stages to adapt to the stressor. Studies that do not take into account effects across multiple life-history stages, especially of organisms with complex life cycles, could seriously under-estimate both the severity of effects and the ability of the organism to adapt. This ability to adapt can also be dependent on the evolutionary history the organism or population (see Gonzalez and Bell 2013) has with the stressor in question. While we saw this clearly with newt larvae and $MgCl_2$, which they have not evolved to regulate (compared to NaCl), this trend is general and important across organisms and stressors. Damselfly larvae from lower latitudes are thermally adapted to warmer temperatures and thus not as impacted by the interacting effects of climate-change induced warming with zinc pollution as populations from higher latitudes, for

example (Dinh Van et al. 2013; Janssens et al. 2014). As the biosphere continues to be impacted by anthropogenic change, organisms will be forced to deal with more and more stressors some of which they have an evolutionary history of regulating (e.g., temperature, UV, NaCl) and others with which they lack this evolutionary history (e.g., pesticides, heavy metals, flame-retardants, alternative deicing salts). Understanding the evolutionary history organisms have with these stressors will be key to assessing the abilities and limitations of adaptation in stressful environments.

Selection events are often unpredictable, episodic and transient in nature -

The nature, frequency, and predictability of environmental perturbations that serve as selection events can have important consequences for evolution in stressful environments (Parsons 2005). Stressful environments are often, by their nature, extremely unpredictable, and selective forces, such as an oil spill, invasive species, or road run-off event, may be introduced without warning at any time (e.g., Whitfield and Wade 1992). These stochastic, episodic selection events introduce very real challenges for organisms and may differ from the more predictable sources of selection that might operate in more "natural" systems. Even more natural stressors, however, such as temperature, which follows a more predictable seasonal cycle, may also play a role in unpredictable, episodic selection. Climate change scenarios forecast increased stochasticity in weather patterns, as well as the frequency of events such as storms (Trenberth 2011), which could act as powerful selection events (e.g., Ch. 7). Understanding how organisms respond to unpredictable episodic selection versus more predictable selection will be key to understanding evolution in stressful environments.

CONCLUSION

This dissertation has explored the broad question of evolution in stressful environments through investigating the evolutionary potential for amphibians to adapt to saline habitats. This has been achieved through both an exhaustive review of the known literature, as well as the collection of original data on all life-history stages of a case-study species, the rough-skinned newt (*Taricha granulosa*). By doing so, I have been able to determine that amphibian populations may be able to adapt through exploiting existing variation in salt tolerance in osmotically stressful, unpredictable environments. While newts have provided critical insights into this topic, it is important to note that most amphibian species have not been examined in an evolutionary light, and we still know very little regarding the genetic nature of salinity tolerance, the variation in tolerance that might exist within populations, and selective pressures, including ecological interactions, and the temporal nature of selection events, which might lead to adaptation. As habitats become increasingly impacted by anthropogenic change, including salinization, it is important to understand what might limit adaptation in amphibians and other organisms, and why some populations or species may struggle to evolve, and/or be constrained by their evolutionary history. This evolutionary perspective, where we seek to understand the factors that regulate the abilities (and constraints) of populations to evolve is critical both in looking back at those "Indian toads…haunting the seaside" (Darwin 1872), and forward at those species facing new stressors, whether they be road deicing salts, landscape modification, or the formation of new seaside haunts as sea levels rise in a changing world.

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APPENDICES

Appendix A: Coauthor Permission Letters

July 27, 2015

Mr. Gareth R. Hopkins
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

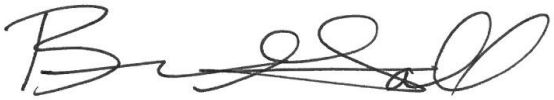
Dear Gareth,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Hopkins, G.R., Gall, B.G., French, S.S. and E.D. Brodie, Jr. 2012. Interfamily variation in amphibian early life-history traits: raw material for natural selection? Ecology and Evolution 2: 1637-1643.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,



Brian G. Gall
Assistant Professor of Biology
Hanover College

July 26, 2015

Mr. Gareth R. Hopkins
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Gareth,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Hopkins, G.R., Brodie, Jr., E.D., Mohammadi, S., Brusch IV, G.A., Neuman-Lee, L.A., Hopkins, Z.M., and S.S. French. Tidal Newts: Physiological responses to an osmotically stressful environment

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Lorin A. Neuman-Lee

Lorin A. Neuman-Lee
PhD Candidate
Department of Biology
Utah State University

July 25, 2015

Mr. Gareth R. Hopkins
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Gareth,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Hopkins, G.R., Brodie, Jr., E.D., Mohammadi, S., Brusch IV, G.A., Neuman-Lee, L.A., Hopkins, Z.M., and S.S. French. Tidal Newts: Physiological responses to an osmotically stressful environment

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Shabnam Mohammadi
PhD Candidate
Department of Biology
Utah State University

July 24, 2015

Mr. Gareth R. Hopkins
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Gareth,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Hopkins, G.R., Brodie, Jr., E.D., Mohammadi, S., Brusch IV, G.A., Neuman-Lee, L.A., Hopkins, Z.M., and S.S. French. Tidal Newts: Physiological responses to an osmotically stressful environment

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

George A. Brusch IV
PhD Candidate
School of Life Sciences
Arizona State University

July 26, 2015

Mr. Gareth R. Hopkins
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Gareth,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Hopkins, G.R., Brodie, Jr., E.D., Mohammadi, S., Brusch IV, G.A., Neuman-Lee, L.A., Hopkins, Z.M., and S.S. French. Tidal Newts: Physiological responses to an osmotically stressful environment

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Zoë Hopkins

Zoë M. Hopkins

Appendix B: Publisher Permission Letters

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PUBLISHING SERVICES

Date: July 29, 2015

Dear Gareth Hopkins,

On behalf of Allen Press Publishing Services, I am pleased to grant permission to you for the reprinting of the following:

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Lindsey Givens
Publishing Specialist
Allen Press Publishing Services

AGREED: Gareth Hopkins DATE: July 29, 2015

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Title: Potential for local adaptation in response to an anthropogenic agent of selection: effects of road deicing salts on amphibian embryonic survival and development

Author: Gareth R. Hopkins,Susannah S. French,Edmund D. Brodie

Publication: Evolutionary Applications

Publisher: John Wiley and Sons

Date: Oct 1, 2012

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Editor: Daniele Canestrelli, Tuscia University, Italy

Received: December 13, 2013; **Accepted:** March 24, 2014; **Published:** April 18, 2014

CURRICULUM VITAE

GARETH R. HOPKINS

Department of Biology and the Ecology Center
Utah State University
5305 Old Main Hill
Logan, UT, U.S.A, 84322

EDUCATION

Degree	Majors	Institution	Major Advisors	Years
Doctor of Philosophy (Ph.D)	- Ecology - Biology	Utah State University	Dr. Edmund Brodie, Jr. Dr. Susannah S. French	2010 - 2015
Bachelor of Science (B.Sc.)	-Biology - Natural Resources Management: Wildlife & Fisheries	University of Northern British Columbia	Dr. Brian Aukema (entomology) Dr. Saphida Migabo (herpetology/ecology)	2005 - 2010

Dissertation Title:
"Salt and the Rough-skinned Newt: Evolutionary Investigations of Local Adaptation to an Anthropogenic and Natural Stressor"

Other Education:
Neotropical Herpetology course, Institute for Tropical Ecology & Conservation, 2009

RESEARCH INTERESTS

- Eco-Evolutionary responses to environmental change, including local adaptation to stressful and novel environments
- Evolutionary biology, ecology, physiology, behavior and conservation of animals, especially those with complex life cycles

RESEARCH EXPERIENCE

2010-15	Graduate Research Assistantship / Teaching Assistantship	Utah State University, Logan, Utah
2007, 2009, 2010	Federal Forest Entomology Student Research Assistant	Natural Resources Canada – Canadian Forest Service / University of Northern British Columbia, Prince George
2008	Amphibian Ecology Research Assistant	Northern Amphibian Monitoring Outpost Society of BC / University of Northern British Columbia, Prince George
2004	Volunteer Research Field Assistant	University of British Columbia, Vancouver, BC *Worked in Dr. Dolph Schluter's evolutionary biology lab, Department of Zoology, studying stickleback speciation.*

PEER-REVIEWED PUBLICATIONS

Hopkins, G.R., French, S.S., and E.D. Brodie, Jr. Interacting stressors and the potential for adaptation in a changing world: Responses of populations and individuals [in preparation]

Spence, A.R.*, **Hopkins, G.R.**, Neuman-Lee, L.A., Smith, G.D., Brodie, Jr., E.D., and S.S. French. The effects of ZnO nanoparticles on egg, larva, and adult rough-skinned newts (*Taricha granulosa*) [in preparation]
*undergraduate mentee

Hopkins, G.R., Brodie, Jr., E.D., Mohammadi, S., Brusch IV, G.A., Neuman-Lee, L.A., Hopkins, Z.M., and S.S. French. Tidal newts: Physiological responses to an osmotically stressful environment. [in review]

Kosmala, G.K.*, **Hopkins, G.R.**, Brodie, Jr., E.D., and S.S. French. Corticosterone levels in larval newts (*Taricha granulosa*) influenced by body size but not salinity. *Copeia* [in review]
*undergraduate mentee

17. **Hopkins, G.R.** and Hopkins, Z.M. 2015. Salty salamander: Occurrence of a *Dicamptodon tenebrosus* in a tidal stream. *Northwestern Naturalist* 96 (2): 147-149.

16. **Hopkins, G.R.** and E.D. Brodie, Jr. 2015. Occurrence of amphibians in saline habitats: A review and evolutionary perspective. *Herpetological Monographs* 29: 1-27.

15. Smith, G.D.**, **Hopkins, G.R**.**, Mohammadi, S., Skinner, H.M., Hansen, T.*, Brodie, Jr., E.D., and S.S. French. 2015. Effects of temperature on embryonic and early larval growth and development in the rough-skinned newt (*Taricha granulosa*). *Journal of Thermal Biology* 51: 89-95.
*** Both authors contributed equally.*
*undergraduate author

14. Neuman-Lee, L.A., Stokes, A.N., Greenfield, S.*, **Hopkins, G.R.**, Brodie, Jr., E.D. and S.S French. 2015. The role of corticosterone and toxicity in the antipredator behavior of the rough-skinned newt (*Taricha granulosa*). *General and Comparative Endocrinology* 213: 59-64.
*undergraduate author

13. Wilson, N.J., Stokes, A.N., **Hopkins, G.R.**, Brodie, Jr., E.D., and C.R. Williams. 2014. Functional and physiological resistance of crayfish to amphibian toxins: tetrodotoxin resistance in the white river crayfish *Procambarus acutus*. *Canadian Journal of Zoology* 92: 939-945.

12. **Hopkins, G.R.,** Brodie, Jr., E.D., and S.S. French. 2014. Developmental and evolutionary history affect survival in stressful environments. *PLoS ONE* 9: e95174. doi:10.1371/journal.pone.0095174.

11. **Hopkins, G.R.,** French, S.S. and E.D. Brodie, Jr. 2013. Potential for local adaptation in response to an anthropogenic agent of selection: effects of road deicing salts on amphibian embryonic survival and development. *Evolutionary Applications* 6: 384-392.

10. **Hopkins, G.R.,** French, S.S., and E.D. Brodie, Jr. 2013. Increased frequency and severity of developmental deformities in rough-skinned newt (*Taricha granulosa*) embryos exposed to road deicing salts (NaCl & $MgCl_2$). *Environmental Pollution* 173: 264-269.

9. Neuman-Lee, L.A.**, **Hopkins, G.R**., Brodie, Jr., E.D., and S.S. French. 2013. Sublethal contaminant exposure alters behavior in a common insect: Important implications for trophic transfer. *Journal of Environmental Science and Health Part B* 48: 442-448.

8. Ferry, E.E.*, **Hopkins, G.R.,** Stokes, A.N., Mohammadi, S., Brodie Jr, E.D. and B.G. Gall. 2013. Do all portable cases constructed by caddisfly larvae function in defense? *Journal of Insect Science* 13: 1-9.
*undergraduate author

7. **Hopkins, G.R.,** Gall, B.G., French, S.S. and E.D. Brodie, Jr. 2012. Interfamily variation in amphibian early life-history traits: raw material for natural selection? *Ecology and Evolution* 2(7): 1637-1643.
**Featured article on* AmphibiaWeb *July 10-15, 2012.* www.amphibiaweb.org

6. **Hopkins, G.R**., Gall, B.G. and E.D. Brodie, Jr. 2011. Ontogenetic shift in efficacy of antipredator mechanisms in a top aquatic predator, *Anax junius* (Odonata: Aeshnidae). *Ethology* 117: 1093-1100.

5. Gall, B.G., **Hopkins, G.R**. and E.D. Brodie, Jr. 2011. Mechanics and ecological role of swimming behavior in the caddisfly larvae *Triaenodes tardus*. *Journal of Insect Behavior* 24: 317-328.

4. **Hopkins, G.R**. and P.N. Lahanas. 2011. Aggregation behaviour in a neotropical dendrobatid frog (*Allobates talamancae*) in western Panama. *Behaviour* 148: 359-372.

3. **Hopkins, G.R.** and S.W. Migabo. 2010. Antipredator skin secretions of the Long-toed Salamander (*Ambystoma macrodactylum*) in its northern range. *Journal of Herpetology* 44: 627-633.

2. **Hopkins, G.R.,** Klingenberg, M.D., and B.H. Aukema. 2009. Temptations of weevil: feeding and ovipositional behaviour of *Hylobius warreni* Wood (Coleoptera: Curculionidae) on bark in laboratory bioassays. *Agricultural and Forest Entomology* 11: 397-403.

1. Öhrn, P.,Klingenberg, M., **Hopkins, G**. and N. Björklund. 2008. Two non-destructive techniques for sex determination of live adult *Hylobius warreni*. *The Canadian Entomologist* 140: 617-620.

CONFERENCE AND INVITED SEMINAR PRESENTATIONS

2015 Annual Meeting of the Society for Integrative and Comparative Biology
Poster Title: "*Effects of temperature on embryonic and early larval growth and development in the rough-skinned newt (Taricha granulosa)"* (Undergraduate author Tyler Hansen presenting)

Oral Presentation Title: "*Newts and nanoparticles: Small particle, big problem?*" (Undergraduate mentee Austin Spence presenting)

Palm Beach, Florida January 4th, 2015

2014 Genomes to Biomes Conference (1st Joint Conference of CSEE, CSZ, and SCL)
Poster Title: *"Tidal Newts: Evolution in a Stressful Environment"*
Montréal, Québec May 25-29th, 2014
*CSEE Poster Award

2014 Annual Meeting of the Society for Integrative and Comparative Biology
Oral Presentation Title: *"Salt and the Rough-skinned newt: Investigations of Adaptation to a Natural and Anthropogenic Stressor."*
Austin, Texas January 5th, 2014
**Co-chair of Evolutionary Physiology Session*

2013 Ecology and Evolution Lunch Seminar Series - McGill University (Invited Seminar)
Oral Presentation Title: Road to sea: Pulses of selection and adaptation in stressful environments (a newt case study).
McGill University, Montréal, Québec December 4th, 2013

2013 Evolution Meeting (Annual Meetings of the Society for the Study of Evolution & American Society of Naturalists)
Poster Title: *"Tidal Newts: Evidence for local adaptation to an osmotically stressful environment"*
Snowbird, Utah June 21-25, 2013

2012 7th World Congress of Herpetology
Oral Presentation Title: *"Embryonic survival in salt among Rough-skinned newt (Taricha granulosa) families"*
University of British Columbia, Vancouver, BC August 8-14th, 2012
*SSAR Seibert Award Honorable Mention

2011 Annual Meeting of the Entomological Society of America
Oral Presentation Title: "*Ontogenetic shift in efficacy of antipredator mechanisms in a top aquatic predator, Anax junius (Odontata: Aeshnidae)*"
Reno, Nevada November 13-16, 2011

2011 Intermountain Graduate Research Symposium
Oral Presentation Title*: "Antipredator behavior of a top aquatic predator: the importance of size and ontogeny in a predatory dragonfly nymph"*
Utah State University, Logan, Utah March 31-April 1st, 2011
*1st Prize (Biology) ($100 prize)

2010 Western Forest Insect Work Conference *(*First Author Laura Machial Presenting*)*
Oral Presentation Title: *I can see clearly now the paint has gone: the role of vision in host selection of Hylobius warreni.*
Flagstaff, Arizona April 5-8th, 2010

2009 Annual Meeting of the Entomological Society of America
Oral Presentation Title: *Temptations of weevil: feeding and ovipositional preferences of Hylobius warreni on bark in laboratory bioassays.*
Indianapolis, Indiana Dec 13-16, 2009

2009 7th Annual UNBC Teaching and Learning Conference
Workshop Title: *Peer-Led Team Learning and the Benefits of Outside of the Classroom Group Learning*
Univ. Northern BC, Prince George, BC September 2-3, 2009

2009 Joint Meeting of Ichthyologists and Herpetologists (JMIH)
Oral Presentation Title: *Antipredator granular gland skin secretions of the long-toed salamander (Ambystoma macrodactylum) in its northern range*.
Portland, Oregon July 22-27th, 2009

2009 Panelist – Provost's Panel on Integrating Teaching and Research
Univ. Northern BC, Prince George, BC November 27th, 2009

2008 Rising Stars of Research National Undergraduate Science Research Competition
Poster Title: *Temptations of weevil: feeding and ovipositional preferences of Hylobius warreni on bark in laboratory bioassays.*
University of British Columbia, Vancouver, BC August 21-23rd, 2008
*1st Prize (Discovery Division [non-medical sciences])

2008 UNBC Natural Resources and Environmental Studies Institute Graduate* Student Poster Presentation
Poster Title: *Temptations of weevil: feeding and ovipositional preferences of Hylobius warreni on bark in laboratory bioassays*
Univ. Northern BC, Prince George, BC October 2008
*Attended as an undergraduate student

FELLOWSHIPS, SCHOLARSHIPS & AWARDS

2015-16	USU School of Graduate Studies Dissertation Fellowship (*declined*)	$5,000.00 USD *(declined)*
2014-15	USU College of Science Graduate Researcher of the Year Award (*Nominee: University-wide Graduate Researcher of the Year ("Robins Award")*)	$300.00 USD
2011-14	Natural Sciences and Engineering Research Council of Canada (NSERC) Postgraduate Doctoral Fellowship	$63,000.00 USD
2014	Cdn. Society for Ecology and Evolution Poster Award (Genomes to Biomes)	$200.00 CD
2014	MacMahon Endowed Ecology Graduate Student Research Award	$1,000.00 USD
2013	Society for Northwestern Vertebrate Biology Student Research Scholarship	$1,000.00 USD
2013-14	USU Ecology Center Graduate Research Support Award	$3,000.00 USD
2012	SSAR Seibert Award (for paper given at World Congress of Herpetology 7)	Honorable Mention
2012-13	USU Ecology Center Graduate Research Support Award	$4,000.00 USD
2012	USU Ecology Center Conference Travel Award to attend WCH7	$700.00 USD
2011	1st Prize (Biology) Presentation Intermountain Graduate Research Symposium	$100.00 USD
2010-11	Natural Sciences and Engineering Research Council of Canada (NSERC) Alexander Graham Bell Canada Graduate Fellowship (*declined in favour of:*)	$17,500.00 CD (*declined)*
	NSERC Postgraduate Fellowship	$17,300.00 CD (*accepted)*

2009	Undergraduate Student Achievement in Entomology Award Entomological Society of America	$2,000.00 USD
2009	SSAR Student Travel Award to attend JMIH Society for the Study of Amphibians and Reptiles	$400.00 USD
2009	UNBC Ecosystem Science and Management Research Travel Award	$200.00 CD
2008	1st Prize (Discovery Division) Rising Stars of Research National Undergraduate Science Research Competition	$500.00 CD
2008	Association of Professional Biologists of B.C. Scholarship	$1,000.00 CD
2008	Petro-Canada Undergraduate Environmental Science Award	$2,000.00 CD
2007	UNBC van Adrichem Family Summer Research Award	$500.00 CD
2005	UNBC Leadership Undergraduate Entrance Award	$2,000.00 CD
2001-03	Medals in Greater Vancouver Regional Science Fairs (*Secondary School*)	

REVIEWER FOR:

Proceedings of the Royal Society B: Biological Sciences
Ecology
Evolutionary Applications
Toxicon
Forest Ecology and Management
Journal of Experimental Zoology A: Ecological Physiology and Genetics
Aquaculture Research
Journal of Thermal Biology
Herpetological Journal
Transactions of the Royal Society of South Australia
Biological Conservation

MEMBERSHIPS IN PROFESSIONAL SOCIETIES

American Society of Naturalists
Society for the Study of Evolution
Canadian Society for Ecology and Evolution
Society for Integrative and Comparative Biology
Canadian Herpetological Society

TEACHING EXPERIENCE

Summer 2015	Instructor	Biology & The Citizen (BIOL 1010)	Utah State University
Spring 2015	Guest Lecturer (2 lectures)	Human Physiology (BIOL 2420)	Utah State University
Spring 2015	Head Teaching Assistant & Lab Coordinator -	Human Physiology (BIOL 2420)	Utah State University
Fall 2014	Guest Lecturer (1 lecture)	Human Physiology (BIOL 2420)	Utah State University
Fall 2014	Teaching Assistant	Human Physiology (BIOL 2420) Laboratory	Utah State University
Fall 2013	Teaching Assistant	Introductory Biology (BIOL 1610) Laboratory	Utah State University
Fall 2011	Teaching Assistant	Introductory Biology (BIOL 1610) Laboratory	Utah State University
2009-10	Peer-led Team Learning Workshop Leader	Introductory Biology I & II (BIOL 101/102)	University of Northern British Columbia
2009	Private Tutor	Grade 12 High School Biology curriculum	Prince George, BC

MENTORING EXPERIENCE

Supervised two independent undergraduate honors research projects, that are now being written/submitted for publication:

- Georgia Kosmala (2012-2013): Effects of salinity on corticosterone levels of larval newts (*Taricha granulosa*)
- Austin Spence (2014-2015): Effects of ZnO nano-particles across life-history stages in an amphibian

Also supervised three other undergraduate research assistant students in lab work (Britain Parish - 2011; Brittany Chamberlain - 2013; Jory Pett - 2012-2014)

ACADEMIC SERVICE

2014-2015	Student Representative, Ecology Faculty Position Search Committee	Utah State University Department of Biology & Ecology Center
2014-2015	Chair, Academic Committee & Member, Fundraising Committee	Utah State University Biology Graduate Student Association
2014	Co-Chair, Evolutionary Physiology Session	Annual Meeting of the Society for Integrative and Comparative Biology
2013-2014	Member, Academic and Fundraising Committees	Utah State University Biology Graduate Student Association
2011-12	President	Utah State University Biology Graduate Student Association
2011-12	Ecology Center Seminar Series Committee Member	Utah State University
2010-11	Vice-President	Utah State University Biology Graduate Student Association
2007	UNBC Student Senator	University of Northern British Columbia Senate
2007-10	UNBC Campus Tour Guide	University of Northern British Columbia Office of Campus Tours

PUBLIC OUTREACH & VOLUNTEER SERVICE

2015	"Call of the Wetlands - Hands on Herpetology"	Bear River Migratory Bird Refuge, Brigham City, UT
2015	Bridgerland Science and Engineering Fair - ISEF Judge	Utah State University
2015	Thomas Edison Elementary School Science Fair - Judge	Thomas Edison Elementary, Nibley, UT
2015	"USU Science Unwrapped" - Herpetology & Entomology Booth	Utah State University
2014	Amphibian Evolution Presentation	Venture Academy High School, Marriott-Slaterville, UT
2014	USU Daycare Herpetology Presentation	Utah State University
2014	"Sense of Wonder Day - Hands on Herpetology"	Bear River Migratory Bird Refuge, Brigham City, UT
2014	Bridgerland Science and Engineering Fair - Judge	Utah State University
2013	"Salamanders are Cool!" Elementary school presentation	Edith Bowen Laboratory School, Logan, UT
2013	"Renewing your Sense of Wonder Day - Hands on Herpetology"	Bear River Migratory Bird Refuge, Brigham City, UT
2012	"USU Science Unwrapped" – Herpetology Booth	Utah State University
2012	"Renewing your Sense of Wonder Day – Hands on Herpetology"	Bear River Migratory Bird Refuge, Brigham City, UT

2012	Intermountain Pow-Wow – USU Biology Department Booth	Utah State University
2011	"USU Science Unwrapped" – Event Organizer: Biology Dept.	Utah State University
2011	"Hands on Herpetology" - USU Women in Science Outreach	Charter Elementary School, Logan, UT
2011	"USU Science Unwrapped" – Entomology Booth	Utah State University
2011	"Hands on Herpetology" Presentation at School Science Fair	Adams Elementary School, Logan, UT
2010	"The Magic of Metamorphosis" Presentation	Children's Choice Daycare, Prince George, BC
2009	"The Magic of Metamorphosis" Presentation	Children's Choice Daycare, Prince George, BC
2009	Adventures in Forestry High School Teaching Module – Forest Entomology	University of Northern British Columbia
2008	Daycare Presentation on Amphibian Biology and Conservation	UNBC Daycare Centre
2007	Trout Fish Hatchery Feeder	Spruce City Wildlife Association, BC
2006	Gallery Interpreter / Animal Care	Exploration Place, Prince George, BC
1998 – 2000	Chair and Founder of Elementary School Environment Committee (*Started school's first recycling program*)	Brighouse Elementary, Richmond, BC

MEDIA COVERAGE

July 10-15, 2012	Feature article on AmphibiaWeb (www.amphibiaweb.org) - Hopkins et al. 2012. *Ecology and Evolution*
September 21, 2008	The Wolf 97FM Prince George Radio Research Interview – *Warren Root Collar Weevil Research*
September 25, 2008	The Prince George Citizen newspaper article: "*Student wins major research competition"*
September 29, 2008	CBC Radio "Daybreak North" Interview- *Warren Root Collar Weevil Research & Concerns to Forestry*
September 25, 2008	UNBC Website Research Feature – "*UNBC Student Captures top award in National Research Competition" (Rising Stars of Research National Undergraduate Science Research Competition)* http://www.unbc.ca/releases/2008/09_25gareth.html
October 3, 2008	Richmond News newspaper article – *"Student wins major research award" (Rising Stars of Research National Undergraduate Science Research Competition)*

REFERENCES

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